THE ACCUMULATION OF THE INFLUENZA VIRUS NUCLEOPROTEIN IN THE NUCLEI OF *XENOPUS* OOCYTES

by

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This thesis is dedicated to Warwick University, who took a chance; to Carol, who is taking a bigger chance; and to my family, who had no choice.

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Declaration

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work of which it is a record has been done by myself, with the exception of those instances where the contribution of others has been acknowleged. All sources of information have been specifically acknowledged by means of reference.

J. Davey

John Davey.

Summary

This work concerns the identification of the information controlling the accumulation of the influenza virus nucleoprotein (NP) in the nuclei of Xenopus oocytes.

The NP accumulates in the nuclei of Xenopus oocytes whether introduced into the oocytes as the protein itself or encoded in RNA or DNA. Since no other influenza virus components are present in the oocytes injected with DNA, this accumulation appears to be a property of the NP itself. In vitro mutagenesis of the cloned NP cDNA has then been used to identify which regions of the protein are important in its nuclear accumulation. Mutant proteins lacking amino acids 327-345 of wild-type NP enter the nucleus but do not accumulate there to the same extent as the wild-type protein, suggesting that this region has a role in nuclear accumulation. This proposed location is strengthened by studies involving the production of fusion proteins in which various amino-terminal sequences of the NP gene are fused to the complete chimpanzee α_1-globin sequence: when globin cDNA is injected into and expressed in oocytes the protein remains in the cytosol, however when the globin cDNA is fused to a portion of NP cDNA which includes the region encoding amino acids 327-345 the resulting fusion protein enters and accumulates in the nucleus. Fusion proteins lacking this region of the NP enter but do not accumulate in the nucleus.

Abbreviations

A	Adenosine
BES	Buffered Earle's saline
BHK-21	Baby hamster kidney cells
bis-acrylamide	N-N'-methylene bisacrylamide
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
CEF	Chick embryo fibroblasts
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
rDNA	Ribosomal DNA
EDTA	Ethylenediaminetetra-acetic acid
FP/R	Fowl plague strain Rostock
G	Guanosine
GMEM	Glasgow's modified Eagle's medium
HA	Haemagglutinin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HSV	Herpes simplex virus
M	Matrix protein
MBS	Modified Barths' saline
MDCK	Madin-Darby canine kidney cells
m.o.i.	Multiplicity of infection
NI	Non-infected
NP	Nucleoprotein

NP40	Nonidet P40
NS	Non-structural
OD	Optical density
P	Polymerase protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pfu	Plaque forming unit
p.i.	Post-infection
poly(A)	Polyadenylated
POPOP	(1,4-bis[2-(5-phenyloxazolyl)]benzene; 2,2'-p-phenylene-bis[5-phenyloxazole])
PPO	2,5-diphenyloxazole
RNA	Ribonucleic acid
mRNA	Messenger RNA
rRNA	Ribosomal RNA
vRNA	Viral RNA
RNase	Ribonuclease
RNP	Ribonucleoprotein
SDS	Sodium dodecyl sulphate
SFV	Semliki forest virus
SRP	Signal recognition particle
SSC	Standard saline citrate
T	Thymidine
TK	Thymidine kinase
Tris	Tris (hydroxymethyl) amino methane
U	Uridine
VSV	Vesicular stomatitis virus

GENERAL INTRODUCTION

SECTION I

INTRACELLULAR PROTEIN TRANSPORT

1. Preamble

The eukaryotic cell can be envisaged as a collection of membrane-delimited compartments surrounded by a plasma membrane. These compartments include the endoplasmic reticulum (ER), Golgi apparatus, mitochondria, nucleus and a variety of other organelles whose presence depends on the cell type. Each compartment contains a characteristic complement of proteins which, apart from the few that are synthesised in the mitochondria, the chloroplasts and (controversially) nuclei, all begin being synthesised in the cytosol. It is therefore necessary for these proteins to be transported from the cytosol to their final destination and this transport necessitates the crossing of at least one cellular membrane. In the last 15 years a great deal of work has led to many insights into the mechanisms of these transmembrane movements and such studies represent one of the few research areas where scientists using prokaryotic and eukaryotic systems co-operate, eukaryotic studies leading the way in biochemistry and prokaryotic studies leading the way in genetics. I would like to begin by discussing the translocation mechanisms in eukaryotic cells and for recent reviews of these processes see Davis and Tai (1980); Kreil (1981); Lodish et al. (1981); Meyer (1982); Sabatini et al. (1982); Strauss and Boime (1982); De Robertis (1983) and Silhavy et al. (1983).

2. The Endoplasmic Reticulum

The secretory pathway was outlined in a series of experiments by Palade and co-workers (see Palade, 1975). Secretory proteins are synthesised in association with the rough endoplasmic reticulum (RER) (Siekevitz and Palade, 1960) before being immediately segregated into the lumen of this organelle (Redman et al., 1966) and subsequently transported out of the cell via the Golgi apparatus and secretory vesicles/granules (Jamieson and Palade, 1967a, 1967b). After the elucidation of this pathway studies of the transport at the molecular level were initiated and led to the formulation of the signal hypothesis (Blobel and Sabatini, 1971). In the ensuing years this hypothesis has been extended and modified to encompass subsequent findings (see Figures 1, 2 and 3). Present beliefs are as follows:

1. Like all proteins synthesised in the cytosol the translation of secretory proteins is initiated on free ribosomes.

2. Translation proceeds until 10 to 40 amino acids of the nascent chain have emerged from the ribosome. At this point the translation of secretory and non-secretory proteins differ (Figure 1a).

3. In secretory proteins there is a specialised amino-terminal signal sequence which directs the binding of a 250,000 dalton RNA/protein complex called signal recognition particle (SRP) (Walter and Blobel, 1981). This complex is cytoplasmic in location and may already be attached to ribosomes (Meyer et al., 1982a). The binding of this complex stops the translation of the secretory mRNA.

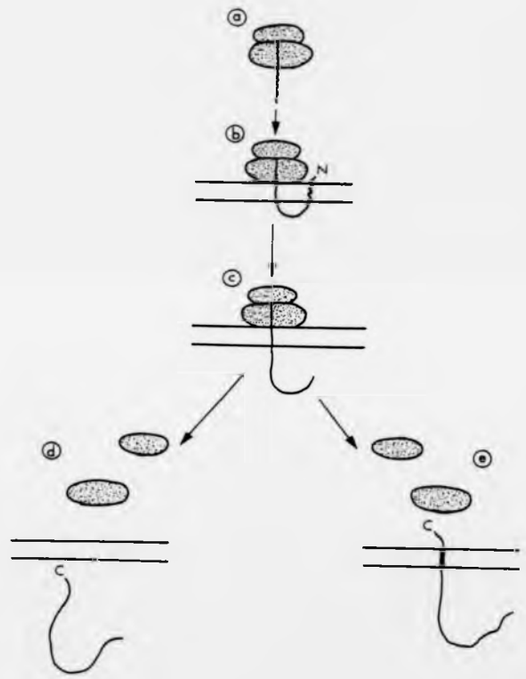


Figure 1 A model of the role of a transient amino-terminal signal sequence in the biosynthesis of secretory proteins and certain integral membrane proteins

The amino-terminal signal sequence emerges from the ribosome (a) and interacts with the ER membrane leading to ribosome attachment and passage of the nascent chain through the membrane (b). The signal sequence is removed by a membrane-associated signal peptidase (c) which leads to the complete translocation of secretory proteins into the ER lumen (d). In transmembrane proteins such as the G protein of vesicular stomatitis virus translocation is stopped by the presence of a stop transfer sequence (e).

4. The translation block persists until the ribosome-SRP-nascent chain complex contacts the RER and binds to an ER specific membrane protein called the docking protein (Meyer and Dobberstein, 1980a, 1980b; Meyer et al., 1982b). This protein releases the block (Meyer et al., 1982a), translation resumes and translocation begins (Figure 1b). The SRP and docking protein dissociate from the translational complex almost immediately.

5. During translocatin, or soon afterwards, the signal sequence is removed by a specific protease called signal peptidase (Figures 1c and 1d).

There are still many questions that remain unanswered concerning some of the details in this process, the main area of debate being the actual movement of the protein through the membrane. The original signal hypothesis predicted the involvement of membrane proteins in the formation of a pore (Blobel and Dobberstein, 1975a, 1975b). Possible candidates for these proteins are two glycoproteins (65,000 and 63,000 daltons) which are prsent in the RER but are apparently absent in smooth membranes (Kreibich et al., 1978a). Since these proteins, termed ribophorins, can be cross-linked to ribosomes (Kreibich et al., 1978b) it has been suggested that they play a role in the binding of the ribosome to the RER. However, smooth ER and RER are equivalent with respect to their in vitro binding of polysomes bearing nascent chains (Bielinska et al., 1979) and these workers also showed that the isolated smooth ER is capable of efficiently translocating and processing secretory polypeptides.

Alternative models, which do not involve membrane proteins mediating the transport of the growing chain, include the 'helical hairpin hypothesis' (Engelman and Steitz, 1981) and the 'direct transfer hypothesis' (von Heijne and Blomberg, 1979; von Heijne, 1980).

As often happens, even with a good hypothesis, there are exceptions and at least one secretory protein, ovalbumin, does not contain a cleavable signal sequence (Palmiter et al., 1978; Chambon et al., 1979). It is hypothesised that there is an uncleaved signal sequence but there is conflicting evidence regarding its position, claims being made for both a central (Lingappa et al., 1978, 1979) and an amino-terminal location (Braell and Lodish, 1982a, 1982b; Meek et al., 1982; Tabe et al., 1984), although not extending to the extreme amino terminus. An uncleaved signal sequence is also found with neuraminidase, a transmembrane protein (Davis et al., 1983; Markoff et al., 1984), and translocation without cleavage has been reported in an in vitro system using the ER of isolated rat pituitaries and preprolactin containing the threonine analogue β-hydroxy-norvaline (Hortin and Boime, 1981).

The ovalbumin data illustrates a further modification of the original signal hypothesis and this involves the transport of the nascent chain through the membrane. The original hypothesis envisaged that transport occurred in a threadlike manner but the ovalbumin results suggest that insertion must occur via a looped formation. These results, and observations on the final disposition of various transmembrane proteins, have also prompted the formulation of alternative models to accomodate the insertion of looped polypeptides into and through the ER membrane (von Heijne and Blomberg, 1979; Wickner, 1980; Engelman and Steitz,

1981). In these models a looped method of insertion has been evoked on the basis of a postulated interaction between the amino-terminal amino acids of the proteins to be transported and the cytoplasmic side of the ER membrane (von Heijne and Blomberg, 1979), or as a means of generating favourable free energies for transfer (Engelman and Steitz, 1981).

Although originally proposed for the translocation of secretory proteins several lines of evidence indicate that the signal hypothesis and associated pathway is also relevant to the transport of most integral membrane proteins of the ER and the plasma membrane. For example, yeast mutants defective in protein secretion are also defective in the incorporation of certain proteins into the cytoplasmic membrane (Novick and Schekman, 1979). During the synthesis of most membrane proteins, for example the glycoprotein (G) of vesicular stomatitis virus (for review see Lodish and Rothman, 1979), the co-translational translocation through the ER membrane is incomplete and the protein remains anchored in the membrane by a short sequence of 20 to 25 hydrophobic amino acids (see Figure 1). This stretch of hydrophobic residues is termed the stop transfer sequence (Blobel, 1980).

As with secretory proteins (e.g. ovalbumin) some integral membrane proteins are synthesised without cleavable signal peptides but are translocated into the ER membrane co-translationally (Figure 2c). These include the ER integral membrane proteins cytochrome P450 (Haugen et al., 1977; Bar-Nun et al., 1980; Kumar and Pamanaban, 1980) and epoxide hydratase (Gonzalez and Kasper, 1980).

Each of the transmembrane proteins described above are simlar in that

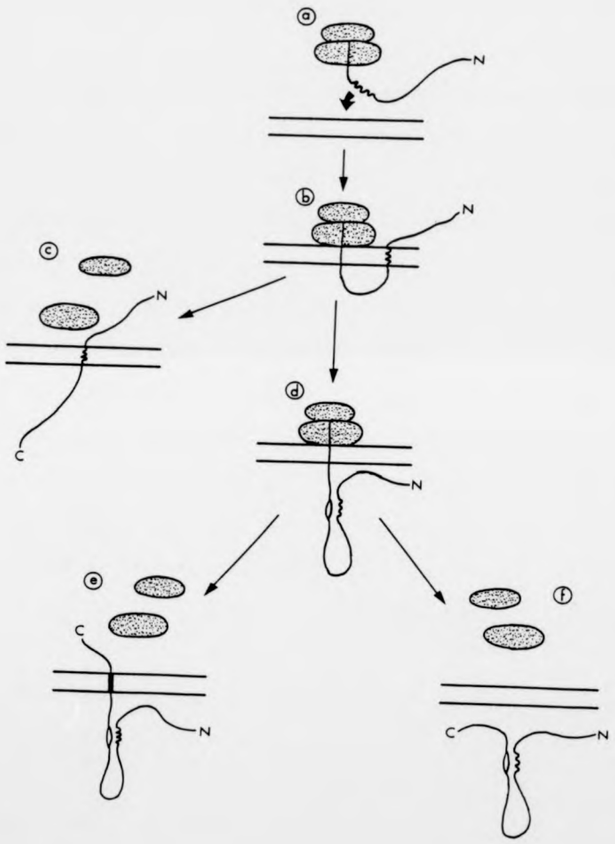


Figure 2 A model of the role of internal permanent signal sequences in the biosynthesis of secretory proteins and certain integral membrane proteins

The affinity of the signal sequence (‡) for the membrane (a and b) may be overcome by interaction with another portion of the polypeptide (ϕ) (d) which facilitates passage of the entire molecule through the membrane (f) unless translocation is interrupted by a stop transfer sequence (e). The protein may remain embedded in the membrane by the signal sequence (c) in which case the topology is the same as that in Figure 3c except that a larger portion of the amino terminus is located on the cytosolic side of the membrane.

they span the membrane once with their carboxyl terminus in the cytosol. Some proteins, for example the influenza virus neuraminidase (Blok et al., 1982) and the intestinal brush border aminopeptidase (Maroux and Louvrd, 1976) and isomaltase (Brunner et al., 1979), are orientated with their amino termini in the cytosol. These proteins are inserted into the membrane co-translationally, but have signal sequences which are not cleaved and are not situated at the extreme amino terminus (Figures 2c and 3). They are therefore similar to ovalbumin except that translocation is incomplete and they are retained in the membrane, usually by the signal sequence. Such sequences have been termed "extended signal sequences" (Frank et al., 1978; Blobel, 1980).

The erythrocyte anion transport protein (Band III) is co-translationally inserted into membranes (Braell and Lodish, 1981, 1982a, 1982b) and spans the membrane at least twice (Steck et al., 1976; Drickamer, 1977; Rao and Reithmeier, 1979; Williams et al., 1979). Such a topology can be explained by proposing multiple signal sequences and stop transfer sequences within a single polypeptide (Figure 3).

All of the integral membrane proteins discussed so far are co-translationally inserted into the ER membrane, although some are synthesised without a cleavable signal sequence. In contrast, cytochrome b5 (Krieter and Shires, 1980; Rachubinski et al., 1980) and NADH-cytochrome b5 reductase (Borghese and Gaetani, 1980) are synthesised on free polyribosomes and inserted into the membrane post-translationally. Both proteins are anchored by a hydrophobic portion at the carboxyl termini with the remainder of the protein in the cytosol. The fact that these proteins become inserted into a variety of cellular

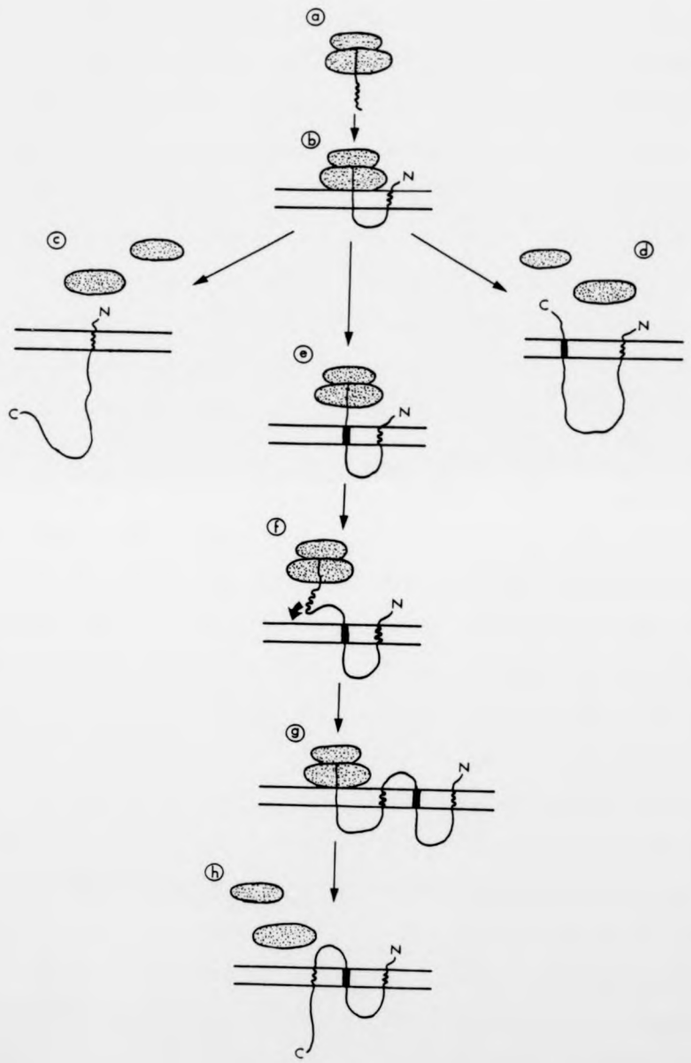


Figure 3 A model of the roles of permanent sequences in the biosythesis of a variety of integral membrane proteins

The amino-terminal signal sequence emerges from the ribosome (a), interacts with the ER membrane and directs the initiation of translocation (b). In the absence of cleavage of the signal sequence the protein remains in the membrane with most of the protein on the luminal side of the membrane (c), e.g. neuraminidase. If the protein contains a stop transfer sequence then it will span the membrane twice with both termini on the cytosolic side of the membrane (d). A third crossing of the membrane may occur if the stop transfer sequence is followed by an interior signal sequence (e-h). Multiple crossings may be generated by alternating interior signal sequences and stop transfer sequences, and the location of the carboxyl terminus depends on whether the last transmembrane signal was a signal sequence or a stop transfer sequence.

membranes and bind to natural and synthetic membranes *in vitro* suggests their insertion is not wholly specific. Such post-translational insertion has led to the formulation of the 'membrane triggering hypothesis' (Wickner, 1979, 1980). Here it is envisaged that the fully folded protein is conformationally changed by association with the correct cellular membrane so that it can cross or become inserted into the membrane. In those proteins containing cleavable signal sequences it is thought that the sequence is important in the correct folding of the protein and its removal is seen as making the membrane interaction irreversible.

From the above discussion it is obvious that compartmentalisation can occur via many different pathways, even for proteins destined for the same location. Similar conclusions resulted from the study of protein transport into cellular organelles.

3. Mitochondria

Mitochondria possess four compartments, the matrix, the inner membrane, the intermembrane space and the outer membrane. The transport of proteins into these various compartments has recently been reviewed (Neupert and Schatz, 1981; Schatz and Butow, 1983) and the major findings are outlined below.

Most of the proteins synthesised in the cytosol and destined for the matrix and the inner membrane of the mitochondrion share common biosynthetic pathways. Following their complete synthesis on free polyribosomes the proteins exist in the cytosol as precursors with amino-terminal extensions (see, for example Neupert and Schatz, 1981). The size of the extension varies considerably among different precursors and is usually much longer than the corresponding prepeptide of secretory proteins. For example, the extension of the ATPase proteolipid subunit of *Neurospora crassa* has 66 amino acids (Viebrock *et al.*, 1982). The precursors appear to bind to receptors on the cytoplasmic face of mitochondria (Henning and Neupert, 1981; Zimmerman *et al.*, 1981) and are transported to their intra-mitochondrial location. Little is known about the transport mechanism but it requires an electrochemical potential across the mitochondrial inner membrane (Gasser *et al.*, 1982; Schleyer *et al.*, 1982), and this could labilise the lipid bilayer (Komor *et al.*, 1979) and facilitate transport via the formation of inverted lipid micelles (Cullis and De Kruijff, 1979). Once at their destination the precursors are converted to their mature forms by a protease located in the matrix (Bohni *et al.*, 1980; Mori *et al.*, 1980; McAda and Douglas, 1982). Some proteins, for example the

adenine nucleotide translocator of the inner membrane (Zimmerman and Neupert, 1980), are synthesised without a cleavable amino-terminal extension but the precursors appear to be conformationally different to the mature proteins.

Transport to the intermembrane space is more complicated than import into the matrix-inner membrane compartment and several mechanisms exist. Cytochrome b2 for example, is synthesised as a 68,000 dalton precursor which is transported across both mitochondrial membranes before being cleaved to an intermediate form (MW = 65,000) by the matrix protease. This intermediate, which probably spans the inner membrane with its bulk in the intermembrane space, is then further cleaved by a second protease to release the mature protein (MW = 58,000) into the soluble intermembrane space (Daum et al., 1982; Gasser et al., 1982). Not all intermembrane space proteins are transported by a two-step pathway however and both adenylate kinase (Watanabe and Kuba, 1982) and the heme-free apoprotein of cytochrome C (Zitomer and Hall, 1976; Zimmerman et al., 1979) are imported without proteolytic cleavage.

Proteins to be imported into the mitochondrial outer membrane are not synthesised as precursors and their transport does not require an energised inner membrane (Freitag et al., 1982).

4. Chloroplasts

It appears that all cytoplasmically synthesised chloroplast proteins share the same biosynthetic pathway regardless of their final

destination (for a review of the structure of chloroplasts see Chua and Schmidt, 1979). All of the proteins studied to date are synthesised with amino-terminal extensions ("transit peptides") which are removed during or soon after their post-translational import into the chloroplast. These include the small subunit of ribulose 1,5-bisphosphate carboxylase (Roy et al., 1976; Dobberstein et al., 1977; Highfield and Ellis, 1978), the δ subunit of the proton translocating ATPase complex (Nelson et al., 1980) and two apoproteins of the pea light harvesting chlorophyll a/b protein complex (Cashmore et al., 1978; Schmidt et al., 1979a, 1979b; Grossman et al., 1980). The transit peptide is different from the signal sequence of secretory and integral membrane proteins as it is generally longer, more hydrophilic and has no long clusters of hydrophobic residues (Palmiter, 1977; Schmidt et al., 1979b). As in some mitochondrial proteins the transit peptide may be cleaved in two stages (C. Robinson and R. J. Ellis, submitted for publication).

Although the above sections imply that protein transport into mitochondria and chloroplasts is post-translational, the demonstration of cytoplasmic ribosomes on the outer membrane of yeast mitochondria (Kellems et al., 1974, 1975) led to suggestions that co-translational transport may also take place. Indeed if the rate of protein synthesis in yeast spheroplasts is reduced by the addition of cycloheximide these mitochondria associated ribosomes are enriched in mRNA encoding for some, but not all, mitochondrial proteins (Kellems et al., 1974, 1975; Ades and Butow, 1980; Suissa and Schatz, 1982). No ribosomes have yet been demonstrated on the outer membrane of chloroplasts (Gunning and Steer, 1975).

5. Lysosomes

Lysosomal enzymes follow similar biosynthetic routes to the majority of secretory and membrane proteins (for review see Hasilik, 1980 and Neufeld and Ashwell, 1980). The pathways seem to diverge at the Golgi apparatus where the majority of lysosomal enzymes are routed to the lysosomes. However, some enzymes are directed to the lysosomes after being secreted and endocytosed. The signals involved in directing lysosomal enzymes to lysosomes are discussed in a later section of the introduction.

6. Peroxisomes

Although these membrane enclosed organelles seem to form by budding of the ER at least two of the enzymes, catalase and uricase, are synthesised on free ribosomes and therefore seem to be, but have not yet been shown to be, incorporated post-translationally (Goldman and Blobel, 1978; Robbi and Lazarow, 1978). It is not known if these proteins are synthesised as larger precursors. The situation is therefore somewhat similar to plant glyoxysomes where malate dehydrogenase is incorporaed post-translationally after being synthesised as a larger precursor (Kawajiri et al., 1972).

7. Nuclei

The majority (possibly all) of nuclear proteins are synthesised in the cytoplasm and are transported into the nucleus post-translationally with no detectable modification of the protein (Gurdon, 1970; Bonner, 1975a, 1975b; De Robertis et al., 1978; Dabauvalle and Franke, 1982; Dingwall et al., 1982). The nucleus is different to the organelles discussed above since it is possible that the proteins do not cross membranes as such but pass through holes ('nuclear pores') in the nuclear envelope.

SECTION II

TOPOGENIC SEQUENCES

The above section has gone some way to describing the mechanisms by which proteins are transported into or across cellular membranes. This section deals with how these proteins are targeted towards the membranes prior to the initial interaction and also how, where relevant, post-translocational transport is controlled. The collective term 'topogenesis' was introduced to describe these targeting procedures (Blobel, 1980). Blobel proposed that the information for intracellular topogenesis resides in discrete topogenic sequences that constitute a permanent or transient part of the polypeptide chain. The number of distinct topogenic sequences has been predicted to be relatively small since many different proteins would be targeted to the same intracellular location (i.e. topologically equivalent). Four types of topogenic sequence have been postulated (Blobel, 1980):

1. Signal sequences

A signal sequence is classically defined as a short stretch (~15-30) of hydrophobic amino acids which are transiently located at the amino terminus of secretory or integral membrane proteins, and direct the vectorial transfer of the polypeptide into or across membranes. For a recent compilation of published signal sequences see Watson (1984). The structure, function and processing of these signals have also been reviewed (Wickner, 1980; Kreil, 1981; Michaelis and Beckwith, 1982). Although the majority of signal sequences conform to this description, in several cases they are neither cleavable (Bos et al., 1984; Julius et al., 1984) nor located at the extreme amino terminus (e.g. ovalbumin and neuraminidase - see earlier).

Proteins which are transported across two membrane bilayers into mitochondria and chloroplasts have also been found to have transient amino-terminal regions (Blobel, 1980; Neupert and Schatz, 1981; Schatz and Butow, 1983). Although these can be considered as signal sequences they are different to those involved in translocation into and across the ER membrane being generally longer and more hydrophilic. These differences are not surprising since they must be clearly distinguished from the 'export' signal sequences and because their transport is initiated only after translation is complete. It is not possible to describe a general structure for the signal sequences of mitochondrial and chloroplast proteins since only a small sample is available and different sequences may be responsible for the targeting of the proteins to the different intra-organellar compartments.

The transport of proteins into nuclei can also be considered to be controlled by a signal sequence (De Robertis et al., 1978; De Robertis, 1983) but, as discussed in more detail elsewhere in the thesis, these sequences are permanent, not necessarily amino-terminal and function post-translationally.

2. Stop transfer sequences

These hydrophobic sequences interrupt the translocation process that was previously initiated by a signal sequence and result in the asymmetric integration of proteins into membranes (Lingappa et al., 1978).

3. Insertion sequences

These sequences interact directly with the lipid bilayer and result in the anchoring of the protein in the membrane.

4. Sorting sequences

These sequences act as determinants for the post-translational movement of proteins from their original site of membrane translocation to their final destination. Proteins with an identical destination may share the same sorting sequences. The most significant site of origin of proteins for post-translocational transport is the ER, which probably supplies proteins to essentially all of the intracellular organelles derived from the ER and also to the plasma membrane (Palade, 1975). Of the four topogenic sequences this is the one for which there is least data and most speculation.

Some sorting sequences are mediated via oligosaccharide side chains. All of the proteins which are inserted through the ER membrane have one or more oligosaccharide side chains added and there are specific instances where the carbohydrate moiety plays an important role in the targeting of proteins to specific destinations. The best example is the phosphomannose recognition system of lysosomal enzymes (for review see Neufeld and Ashwell, 1980).

During their transport through the Golgi apparatus lysosomal enzymes acquire mannose-6-phosphate residues by a two-step reaction (Waheed et al., 1981b; Pohlman et al., 1982; Goldberg and Kornfeld, 1983). The first step is the addition of N-acetylglucosamine-1-phosphate to the C6 hydroxyl position of one or two mannose residues (Varki and Kornfeld, 1980b; Hasilik et al., 1981; Reitman and Kornfeld, 1981). The enzyme responsible for this reaction appears to recognise both the carbohydrate and protein moieties (Waheed et al., 1982), suggesting that there may be a common protein sorting sequence in lysosomal enzymes (Fischer et al., 1980; Waheed et al., 1982). In the second step the mannose-6-phosphate residues are exposed (Varki and Kornfeld, 1980a, 1981; Waheed et al., 1981a). This results in a specific interaction with a mannose-6-phosphate receptor in the Golgi apparatus which leads to sequestration of the lysosomal enzymes and ultimately their transport to lysosomes (Fischer et al., 1980; Varki and Kornfeld, 1981). Failure to add the recognition marker to lysosomal enzymes, as in I-cell disease (Hasilik and Neufeld, 1980; Hasilik et al., 1981; Reitman et al., 1981), leads to the secretion of recognition defective enzymes (Hickman et al., 1974). Thus although localisation is mediated via mannose-6-phosphate residues the information appears to ultimately reside in a sorting sequence within the enzyme. This sequence has not been identified.

The biosynthetic pathways of lysosomal enzymes and 'export' proteins diverge at the Golgi apparatus and it has been suggested that this organelle, at which all glycoproteins seem to spend some time, plays an important role in directing many proteins to their final destinations (Palade, 1975; Tartakoff, 1980; Farquhar and Palade, 1981; Rothman et al., 1981). Indeed, in the case of secretory proteins, there is now

direct evidence for two distinct pathways which probably diverge at the Golgi apparatus and are presumably controlled by sorting sequences (Gumbiner and Kelly, 1982). The first pathway, which occurs in most, if not all, cell types, mediates an apparently continuous and non-concentrative exocytosis of secretory proteins while in the second pathway newly synthesised glycoproteins are concentrated and stored in intracellular granules. These granules can be induced to fuse with the plasma membrane by specific secretagogue stimuli. The information controlling these pathways is currently being investigated by Kelly and co-workers.

The transport of lysosomal enzymes from the Golgi apparatus to lysosomes appears to occur via clathrin-coated vesicles (Friend and Farquhar, 1967; Nichols et al., 1971; Campbell and Rome, 1983; Campbell et al., 1983). These vesicles also appear involved in the intracellular transport of proteins from the RER to the Golgi body (Palade and Fletcher, 1977), through the Golgi apparatus (Palade, 1975; Rothman and Fine, 1980; Rothman et al., 1980; Farquhar, 1981; Rothman, 1981), and from the Golgi body to the plasma membrane (see Brown et al., 1983). They are also involved in protein transport in the opposite direction, i.e. the uptake of proteins from the extracellular medium (receptor mediated endocytosis) (for reviews see Goldstein et al., 1979; Pastan and Willingham, 1981). It seems therefore that coated vesicles play a central role in the transport of glycoproteins through the cytoplasm and in order for them to perform this role effectively they must also play a part in directing proteins to specific organelles. In view of this, it has been suggested that coated vesicles may be involved in sorting (Pearse and Bretscher, 1981; Rothman et al., 1981). Proteins with the

correct sorting sequence would presumably be recognised by a specific receptor protein, either in the lumen of the RER or the Golgi apparatus or at the plasma membrane, and taken up into a coated vesicle. After uncoating, the vesicle is directed to a specific organelle by means of a second signal on its cytoplasmic surface. This targeting may be mediated through random interactions between the vesicles and target organelles (Rothman et al., 1981), which would require a 'docking' protein in the membrane of the target organelle, or it may be achieved by the vesicles being directly targeted to specific organelles by some sort of 'tram-line' system (Jamieson, 1981; Pastan and Willingham, 1983; Wehland and Willingham, 1983). Although this targeting is presumably controlled by sorting signals within the proteins they remain unidentified.

SECTION III

ANALYSIS OF TOPOGENIC SEQUENCES

If topogenic sequences are defined stretches of amino acids within a polypeptide chain then mutation of this region should alter the fate of the protein. Most of the experiments of this type have been performed using bacteria and so before describing these experiments I will briefly describe the protein localisation mechanisms in bacteria. For reviews of bacterial transport and its manipulation see Emr et al. (1980a, 1980b), Kreil (1981), Michaelis and Beckwith (1982) and Silhavy et al. (1983).

1. Protein transport in bacteria

The cytosol of Escherichia coli is bounded by two membranes which define three extra-cytoplasmic compartments; the inner membrane, the outer membrane and the intermembrane or periplasmic space. Most proteins destined for these compartments are synthesised on membrane-bound ribosomes as precursors with amino-terminal extensions which are removed during or soon after their co-translation translocation (Canceeda and Schlessinger, 1974; Randall and Hardy, 1977; Smith et al., 1977, 1979, 1980). This similarity with the eukaryotic signal hypothesis extends even to allowing interchangeability between the systems, eukaryotic proteins being processed and exported from prokaryotic cells (Fraser and Bruce, 1978; Talmadge et al., 1980) and prokaryotic proteins being processed by eukaryotic membranes in vitro (Roggenkamp et al., 1981; Müller et al., 1982). Evidence regarding post-translational transport in prokaryotes is contradictory. For example, although the β-lactamase precursor can be found in the cytosol (Koshland and Botstein, 1980) the

proposed post-translational transport to the periplasmic space is difficult to reconcile with results obtained from *in vitro* systems derived from eukaryotes (Müller *et al.*, 1982) and also with results obtained using fusion proteins (Talmadge *et al.*, 1980) and mutation analysis (Koshland and Botstein, 1980; Koshland *et al.*, 1982). Even in *in vitro* systems derived from prokaryotes, membrane vesicles must be added very early to observe any transport (Goodman *et al.*, 1981; Watts *et al.*, 1981). A possible explanation is provided by the recent work of Randall (1983) who showed that, although transport proteins are synthesised in association with membranes, the time at which they are transported is variable. Some proteins are transported in a co-translational manner analogous to the eukaryotic situation while others are only transported when all or nearly all of the protein has been produced.

Although all of the characterised periplasmic and outer membrane proteins and most of the inner membrane proteins contain an amino-terminal signal sequence that is removed during the export process, several inner membrane proteins are not made in precursor form. These include the lactose permease (Ehring *et al.*, 1980), the histidine permease (Higgins *et al.*, 1982) and two subunits of the Fo complex of ATPase (Nielsen *et al.*, 1981). Although these proteins may contain a non-cleavable signal sequence they are highly hydrophobic and may simply partition into the membrane after synthesis, i.e. they may contain sequences that are functionally equivalent to insertion sequences in the signal hypothesis.

2. Signal sequences

The importance of the signal sequence in initiating the co-translational interaction between proteins and membranes has been demonstrated by both mutating (see Silhavy et al., 1983) and removing (Gething and Sambrook, 1982) this region in a variety of proteins. The mutagenesis of prokaryotic signal sequences has allowed the further identification of those features of the signal such as the positions of hydrophobic and hydrophilic residues which are necessary for this mediation (for review see Silhavy et al., 1983). In the majority of cases the signal sequence is apparently also sufficient for this interaction between proteins and membranes. This has been shown in both prokaryotes (Bremer et al., 1980, 1982; Ito and Beckwith, 1981; Boeke and Model, 1982; Koshland et al., 1982) and eukaryotes (Sveda and Lai, 1981; Gething and Sambrook, 1982; Rose and Bergman, 1982; Sveda et al., 1982). In some situations however a signal sequence is not sufficient for the export of a protein from the cytoplasm (Moreno et al., 1980) and other sequences within the protein are involved.

The mutation of signal sequences responsible for transport into mitochondria and chloroplasts has yet to be reported, while the mutation of signals involved in the accumulation of proteins in nuclei is described elsewhere in the thesis.

3. Stop transfer sequences

Removal of the hydrophobic stop transfer sequence from both prokaryotic (Boeke and Model, 1982) and eukaryotic (Sveda and Lai, 1981; Gething and Sambrook, 1982; Rose and Bergman, 1982; Sveda et al., 1982) transmembrane proteins results in their complete translocation across the relevant membrane. For bacterial inner membrane proteins this causes export to the periplasm while for the eukaryotic proteins it leads to their secretion.

A similar situation exists naturally in eukaryotic cells where, as a result of different RNA splicing events, the gene encoding the μ-chain of immunoglobulin M (IgM) can direct the synthesis of both a secreted and membrane-bound form of the protein (Alt et al., 1980; Early et al., 1980; Kehry et al., 1980; Rogers et al., 1980). The only difference between the two forms is at the carboxyl terminus where the secreted form has a hydrophilic sequence while the membrane-bound form has a hydrophobic sequence which is believed to retain the protein in the membrane. The stop transfer capability of this hydrophobic sequence was demonstrated by the construction of a series of plasmids encoding transmembrane proteins from plasmids which previously encoded secreted proteins (Yost et al., 1983). The sequence for the transmembrane portion of the IgM heavy chain was inserted between the coding regions for β-lactamase and globin in a fusion protein. Whereas the protein had previously been translocated across microsomal membranes in a cell-free transcription-linked translation system it was now retained in the membrane (Yost et al., 1983).

4. Sorting sequences

As mentioned earlier there is little information available regarding the identification of sorting sequences. There are a number of examples however where an alteration in the sequence of a protein leads to a change in its transport pathway. In eukaryotic cells the majority of these examples involve proteins that are synthesised at the ER. Mutation of the cytoplasmic tail region of the G protein of vesicular stomatitis virus, for example, leads to the accumulation of the protein in the ER membrane (Rose and Bergman, 1983). This is not a general phenomenon however since mutation of the cytoplasmic tail of the E2 protein of Semliki Forest virus (Garoff et al., 1983) and mouse Class I antigens (Zuniga et al., 1983) has no effect on either the surface expression or the function of these proteins.

As is always possible in such analysis the mutation of a protein may change its conformation and thereby influence the results. For example, a conformational change arising from a single amino acid change is invoked to explain a variant MOPC 315 immunoglobulin light chain which is capable of entering the ER lumen but fails to be secreted from both myeloma cells (Mosmann et al., 1979) and Xenopus oocytes (Valle et al., 1983).

It appears that in the case of imported mitochondrial proteins, different proteins may use different polypeptide regions for targeting. It has been reported that the carboxyl-terminal region of a yeast mitochondrial outer membrane protein is not necessary for its

localisation (Reizman et al., 1983), however, it is necessary for the correct localisation of apocytochrome C to the intermembrane space (Matsuura et al., 1981).

SECTION IV

DISTRIBUTION OF PROTEINS BETWEEN THE CYTOPLASM AND THE NUCLEUS

The above sections reveal that although a great deal is known about the mechanisms of intracellular protein transport there are many gaps in our knowledge. Until recently one of these largest gaps was how proteins were transported into and accumulated in cell nuclei, and it is this area which this thesis is concerned with. Based on the distribution of a protein with respect to the cytoplasm and the nucleus it is possible to distinguish three classes of endogenous proteins in Xenopus oocytes (Gurdon, 1970; Bonner, 1975a, 1975b; De Robertis et al., 1978) and, presumably, other eukaryotic cells. There are those proteins which are found in both the cytoplasm and the nucleus and there are those which are either predominantly cytoplasmic or nuclear in location. The following is a more detailed discussion of each of these classes.

1. Proteins found in both the cytoplasm and the nucleus

These include some abundant soluble proteins such as the soluble forms of actin (Clark and Merriam, 1977; De Robertis et al., 1978) and certain chromatin constituents such as protein HMG-1 (Rechsteiner and Kuehl, 1979; Kleinschmidt et al., 1983). The equal distribution of soluble actin has been shown by the fractionation of radioactively labelled Xenopus oocytes (De Robertis et al., 1978) and by studies of the movement of iodinated actin injected into these cells (De Robertis et al., 1983). Similar results with Amoeba proteus has been interpreted as suggesting that the equal distribution of actin was due to the free movement of the protein across the nuclear envelope (Goldstein et al., 1977). It should be noted that not all of the actin in a cell is

diffusible and in Xenopus oocytes about 25% of the nuclear actin is stably associated with an insoluble nuclear gel (Clark and Merriam, 1977).

2. Proteins found predominantly in the cytoplasm

This class describes those proteins which, after synthesis, remain in the cytoplasm and are excluded from the nucleus, or at least are maintained at a much higher concentration in the cytoplasm. This is a controversial class of proteins since the exclusive localisation of certain proteins to the cytoplasm either after the fractionation of radioactively labelled Xenopus oocytes (De Robertis et al., 1978) or following the micro-injection of radioactively labelled proteins into these cells (Bonner, 1975b; De Robertis et al., 1983) might be explained by their assembly into, or association with, large particles (see Goldstein and Ko, 1981). For example, the cytoplasmic localisation of tubulin (De Robertis et al., 1978, 1983) could be explained by its inclusion into microtubules.

Better examples of this class of proteins have recently been described (De Robertis et al., 1983; Dabauvalle and Franke, 1984). The first is a group of five proteins which bind to U-Sn RNAs (cited in De Robertis et al., 1983). In the absence of U-Sn RNA the proteins are stockpiled in the cytoplasm and are excluded from the nucleus. When U-Sn RNA becomes available, for instance by the injection of the purified RNA, the proteins bind to the RNA and are transported into the nucleus

(Zeller et al., 1983). Dabauvalle and Franke (1984) have recently identified several soluble proteins which apparently remain enriched in the cytoplasm or both Xenopus oocytes and HeLa cells. These include some of the most abundant soluble proteins in these cells and many are of a size that is estimated to be able to pass freely through the nuclear pores (see Section V).

3. Proteins found predominantly in the nucleus

These are proteins which, soon after their synthesis in the cytoplasm, rapidly migrate into and accumulate in the nucleus. Such proteins are called karyophilic proteins and they are often found to be more than 100-fold more concentrated in the nucleus than in the cytoplasm (Krohne and Franke, 1980b; Mills et al., 1980; Feldherr et al., 1983). The ability of these proteins to accumulate in nuclei has been demonstrated by their injection into the cytoplasm of Xenopus oocytes (Gurdon, 1970; Bonner, 1975a, 1975b; De Robertis et al., 1978; Mills et al., 1980; Dabauvalle and Franke, 1982; Dingwall et al., 1982).

SECTION V

TRANSPORT OF PROTEINS ACROSS THE NUCLEAR ENVELOPE

1. Structure of the nuclear envelope

Most, if not all, proteins which accumulate in the nucleus appear to cross the nuclear envelope and so this section describes the basic structure of this barrier. Since the amount of information regarding the structure of the nuclear envelope is so great only the major findings are described here and for a more extensive treatment of the subject the reader is referred to the reviews of Franke (1974a), Franke and Scheer (1974), Kasper (1974) and Fry (1976, 1977).

The existence of a membranous boundary between the nucleus and the cytoplasm had been postulated for a long time before being confirmed by the use of light and interference microscopy (Baud, 1948). The boundary does not constitute a membrane as such, and so is more correctly referred to as the nuclear envelope (Anderson, 1953). It consists of two roughly parallel lipid bilayers, one of which is in contact with the nucleoplasm (inner membrane) while the other is in contact with the cytoplasm (outer membrane). The two membranes are separated by a gap of about 150-300 Å called the perinuclear space (Policard and Bessis, 1956). This contains neither cytoplasm nor nucleoplasm and is presumably filled with a fluid similar to that contained in the cisternae of the endoplasmic reticulum (see below). It has very little distinctive structure although there have been reports of fibers crossing the space and connecting the two membranes (see, for example, Franke et al., 1973). These fibers may be involved in protecting the nuclear envelope during osmotic shock.

The early view of the nuclear envelope as being a distinct membranous boundary has now been replaced with one of it being a specialised interface interacting with both the cytoplasm and the nucleoplasm. Many cytoskeletal elements run parallel to, and often bind to, the outer membrane and these are believed to be involved in determining the shape of the nucleus and also in anchoring the nucleus within the cell (Franke and Scheer, 1974; Osborn and Weber, 1977; Lehto et al., 1978). The outer membrane has ribosomes studded along much of its cytoplasmic face and at several points it is continuous with the endoplasmic reticulum (Palade, 1955; Watson, 1955). Lining the inner aspect of the envelope is an electron-dense layer that has been alternatively referred to as the fibrous lamina (Fawcett, 1966), the zonula nucleum limitans (Patrizi and Poger, 1967) and the internal dense lamella (Stevens and André, 1969), although the preferred term nowadays is the nuclear lamina (Kaufmann et al., 1981). It appears to be composed of homogeneous, finely granular material which is predominantly proteinaceous in content (Bronens and Kasper, 1973; Jackson, 1976; Shaper et al., 1979). The thickness of the lamina is usually 150-200 Å although it is species, tissue and cell cycle dependent (Fawcett, 1966; Oryschak et al., 1974). In some cell types no lamina is visible by electron microsocopy, but the ability to isolate corresponding structures from these cells (see, for example, Aaronson and Blobel, 1975) suggests that the lamina is a ubiquitous feature of nuclei.

At irregular intervals the outer and inner membranes fuse to leave small gaps in the envelope where there is no membranous element between the nucleoplasm and the cytoplasm. Structural and dynamic studies indicate

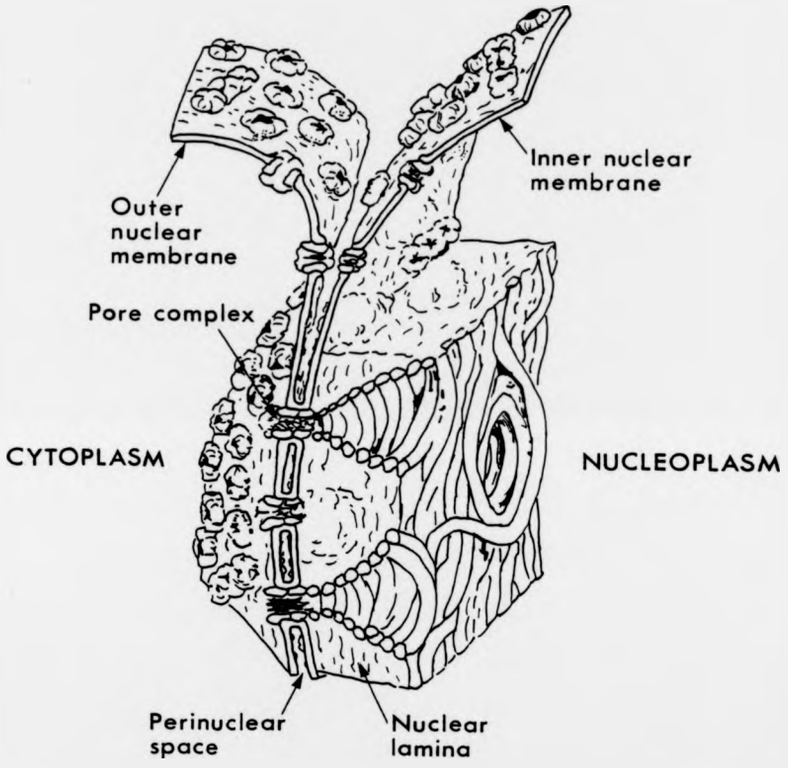


Figure 4 Basic features of the nuclear envelope

that these gaps are not holes as such but are filled with electron opaque material called annular material and have subsequently been termed nuclear pores. The pores are circular or octagonal in appearance and are extensively connected to the nuclear lamina (Aaronson and Blobel, 1975; Berezney and Coffey, 1976). The number of pores per unit area of nuclear envelope is variable and depends on the type of cell being studied, its metabolic state and also its stage of division (Franke and Scheer, 1974; Wunderlich et al., 1974; Zerban and Werz, 1975; Schel et al., 1978). In electron micrographs the pore complex, i.e. the pore and its associated material, appears as a hollow cylinder extending through the envelope and to a distance of about 200 Å on either side. The central channel is about 150-200 Å in diameter and often contains a central granule. The staining properties of this granule (Abelson and Smith, 1970) and its sensitivity to RNase treatment (Mentreé, 1969) suggest it may contain ribonucleoprotein but it is unclear whether this represents a particle in transit or is a genuine structural element of the pore complex. This point is discussed in more detail elsewhere in the introduction (see page 48). As yet no enzymic activity has been conclusively ascribed to the pore complex and although an ATPase activity has been tentatively located there (Yasazumi and Tsubo, 1966; Yasazumi et al., 1967) the situation is unclear (Vorbrodt, 1974; Clawson and Shuckler, 1979). For a more exhaustive review of nuclear pores see Maul (1977). Figure 4 illustrates the basic features of the nuclear envelope.

2. Transport of non-nuclear proteins across the nuclear envelope

In the last 20 years there have been many studies on the permeability of the nuclear envelope. The technique employed in the majority of these studies involves the micro-injection of substances with known properties into the cytoplasm of a cell and then monitoring their entry into the nucleus. Such studies led to the conclusion that the principle factor governing the nuclear entry of a substance that is not naturally found in nuclei is its size. This is probably best illustrated by the work of Paine and co-workers who used cryofixation and autoradiography to monitor the nuclear entry of tritiated dextrans following their injection into the cytoplasm of Rana pipiens oocytes (Paine et al., 1975). In an aqueous environment the dextrans behave as spheres (Grotte, 1956) and by using dextrans of different lengths it is possible to generate a series of molecules which differ only in their size. From the rates with which the different dextrans entered the oocyte nucleus it was possible to calculate that the nuclear pores have a permeant radius of about 45 Å (Paine et al., 1975). Such a value not only substantiates the earlier semi-quantitative studies using similar tracers (Paine and Feldherr, 1972; Horowitz et al., 1973; Feldherr, 1974), but also agrees with results obtained following the injection of non-nuclear proteins into the cytoplasm of these cells. (The term non-nuclear is used to describe proteins which do not accumulate in nuclei). The use of proteins in these studies is complicated by the fact that the majority are aspherical and some, for example γ-globulin, are polymeric and can complicate interpretation by dissociating within the cytoplasm (for example see Bonner, 1975a). It is possible, however, to use the 45 Å pore model to distinguish three classes of non-nuclear proteins.

a) Proteins where the longest molecular dimension is less than 90 Å

When these proteins are injected into the cytoplasm of amphibian oocytes they rapidly enter the nucleus and become evenly distributed between this compartment and the cytoplasm within 24 h. Within this class the rate with which a protein enters the nucleus is inversely proportional to its size (Paine and Feldherr, 1972; Bonner, 1975a).

b) Proteins with one molecular dimension less than 90 Å

These proteins exhibit a slow but definite entry into the nucleus following their injection into the cytoplasm (Gurdon, 1970; Bonner, 1975a). The most conspicuous example of this group is bovine serum albumin (BSA) which has a molecular weight of 67,000 and molecular dimensions of 150 x 38 Å (Young, 1963). After injection into the cytoplasm of Xenopus oocytes BSA enters the nucleus but is still not evenly distributed between the nucleus and the cytoplasm after 72 h. A possible explanation for the slow rate of nuclear entry of these proteins is that they can only pass through the nuclear pores in an 'end-on' configuration.

c) Proteins where the shortest molecular dimension is more than 90 Å

Proteins of this size, for example ferritin which has a molecular diameter of 95 Å, do not enter the nucleus following injection into the cytoplasm (Gurdon, 1970; Paine and Feldherr, 1972; Bonner, 1975a).

Initially, for technical reasons, such direct demonstrations were limited to amphibian oocytes and other large cells such as cockroach oocytes (Paine and Feldherr, 1972) and the salivary gland cells of Chironomus thummi (Paine, 1975). However, because of the extraordinary conservation of the structure of the nuclear envelope between phylogenetically distant species (Franke, 1970; Feldherr, 1972; Harris et al., 1974), it was assumed that these results would be typical of most cells. This assumption was proved correct following the development of a method for the micro-injection into HeLa cells (Stacey and Allfrey, 1976). Although the time scale for transport to the nucleus was much shorter than in the large cells, probably as a consequence of the shorter path-length from the site of injection to the nucleus, the HeLa nuclei were found to exclude proteins on a similar basis.

Recently the technique of fluorescence microphotolysis has been used to estimate the permeant radii of nuclear pores in rat liver nuclei (Peters, 1983). These pores were found to have a permeant radius of about 56 Å, and the small difference between this and the estimate from amphibian oocytes may reflect slight species dependent variations which are also observed by electron microscopy (Fry, 1977).

Although the above experiments primarily monitor movement from the cytoplasm to the nucleus, the fact that many injected particles rapidly equilibrate between the two phases suggests that their exit from the nucleus follows the same mechanism as their entry. The ability of a variety of proteins to equilibrate between the cytoplasm and the nucleus

at the same rate regardless of their injection site, shows this suggestion to be correct (Paine, 1975; Stacey and Allfrey, 1976).

Whereas the size of a protein greatly influences its rate of transport across the nuclear envelope, its charge has little or no effect. Acidic, neutral and basic proteins have all been shown to enter nuclei at rates predicted solely on their molecular dimensions (Paine and Feldherr, 1972; Bonner, 1975a; De Robertis et al., 1978).

3. Transport of nuclear proteins into nuclei

As discussed above the most important feature of non-nuclear proteins with regard to their ability to cross the nuclear envelope is their size. This observation, however, does not apply to proteins normally found in nuclei.

On the basis of the above work proteins larger than ovalbumin (44,000 daltons) should enter nuclei very slowly while proteins larger than BSA (67,000 daltons) should not enter nuclei at all. One might therefore predict that there should be a maximum size limit on nuclear proteins of about 60-70,000 daltons. Many nuclear proteins, however, are considerably larger than this. Xenopus oocyte nuclei, for example, contain a number of proteins in excess of 110,000 daltons (Feldherr, 1975; De Robertis et al., 1978; Mills et al., 1980), while one of the major nuclear proteins of Rana oocytes has a molecular weight of 148,000 daltons (Feldherr et al., 1983). The following sections discuss a

number of ways in which such large proteins may find themselves in nuclei.

a) Influence of molecular shape

One insight as to how large proteins may pass through nuclear pores comes from electron micrographs apparently showing ribonucleoproteins in transit from the nucleus to the cytoplasm (Takamoto, 1966; Monneron and Bernhard, 1969; Franke and Scheer, 1970b). These particles seem to pass through the nuclear pore by unfolding and constricting to a radius of about 50 Å even though they may be up to 1000 Å across on either side of the pore. By analogy to this a large nuclear protein may pass through pores by constricting itself or by already being rod shaped. A good example of this is myosin which consists of two subunits of 234,000 daltons and yet enters HeLa cell nuclei at the same rate as ovalbumin which has a molecular weight of 44,000 daltons (Stacey and Allfrey, 1976). The important consideration here appears to be the rod-like configuration of myosin with its molecular dimensions of 435 x 29 Å.

Further evidence for proteins entering nuclei in an end-on configuration is provided by certain steroid receptors. When steroid hormones such as progesterone enter cells they bind to a receptor protein in the cytoplasm to form a protein-steroid complex. This complex becomes activated by the binding of a third protein termed protein X, and enters the cell nucleus by an apparently passive diffusion process (Shyamala and Gorski, 1969; Gorski and Raker, 1973). The majority of such activated complexes are larger than would be expected to enter nuclei

under the considerations described earlier, but most have high axial ratios. The activated progesterone receptor, for example, has a molecular weight of 90,000 daltons but an axial ratio of 18:1 (Sherman et al., 1970).

It therefore seems conceivable that at least some large nuclear proteins could gain entry through nuclear pores by having a rod-like configuration. The transport of these proteins would be greatly facilitated if there was some form of interaction between the protein and the pore complex which resulted in the protein being presented to the pore in an "end-on" manner. A similar interaction between the protein and the nuclear pore complex forms the basis of the second proposed means of entry.

b) Change in the permeant radius of the nuclear pore

Since the molecular weight of a spherical protein increases as a function of the cube of its radius, a large increase in molecular weight is accompanied by a relatively small increase in molecular size. Similarly a small increase in the permeant radius of the nuclear pore would have a profound effect on its ability to allow the passage of large proteins. For an in depth discussion of this point see Paine et al. (1975).

It is possible therefore that proteins which are too large to pass through a nuclear pore having a permeant radius of 45 Å enter the

nucleus by interacting with the pore complex in such a way as to increase the permeant radius. Although there is no direct evidence to support this model the pore complex does seem to have the potential to accomodate such an increase since the permeant radius of 45 Å is remarkably small compared to the inner pore radius of about 350 Å (Gall, 1967; Franke and Scheer, 1970b).

The above mechanisms both assume that the passage of proteins across the nuclear envelope occurs via the nuclear pores. I think it is therefore appropriate at this point to briefly review the evidence suggesting that the nuclear pores constitute a route across the nuclear envelope. There are principally three lines of evidence implicating pores in transport across the nuclear envelope.

The first of these has been discussed in detail elsewhere and concerns the fact that following their injection into the cytoplasmic regions of cells, the observed nuclear transport of a variety of substances was correlated with their ability to pass through holes with a radius of 45 Å (Paine et al., 1975).

Further evidence comes from electron microscopy showing material apparently in transit across the nuclear envelope. Gold particles injected into amoebae, for example, are often seen in nuclear pores (Feldherr, 1962a, 1965), while studies of virus-infected cells suggest that certain viruses use the pore as an entry route for their nucleic acid (Morgan et al., 1969; Summers, 1969, 1971; Chardonnet and Dales, 1970), and as an exit route for structures produced during infection

(Bates et al., 1974). Although these results may be due to the perturbation of the cell several studies of non-manipulated cells also show structures apparently in transit through pores (Tokamoto, 1966; Monneron and Bernhard, 1969; Franke and Scheer, 1970b). These structures contain ribonucleoprotein and the results have led to suggestions that the central granule observed in some pores is not a component of the pore complex but represents these particles in transit. Indeed, the staining properties (Abelson and Smith, 1970) and RNase sensitivity (Mentré, 1969) of the granules indicate a ribonucleoprotein containing structure. The fact that the percentage of pores containing granules increases with an increased synthetic activity of the nucleus (Merriam, 1962; Comes and Franke, 1970; Franke and Scheer, 1970b; Wunderlich and Speth, 1972), also indicates a transitory structure although it is possible that a low activity means that the granules are more readily lost during isolation or are more difficult to see due to a change in their structure (Wunderlich, 1969; Scheer, 1970).

Indirect evidence for the involvement of pores in nuclear transport comes from the general correlation between the frequency of occurrence of the pores and the need for the exchange of macromolecules across the nuclear envelope. In developing oocytes, for example, where there is a high rate of protein synthesis which presumably requires a steady flow of materials across the nuclear envelope, there is a high pore frequency (Krauskopf, 1968; Szollosi et al., 1972). As the oocytes mature the level of synthetic acitivity decreases and so does the pore frequency (Szollosi et al., 1972). Similar correlations are also seen during the development of the mammalian erythroid series (Grasso et al., 1962), and

during the phytohaemagglutinin stimulation of lymphocytes (Maul et al., 1972).

Although it seems that the pores represent the most likely route across the nuclear envelope a number of other routes can be imagined.

c) Transport via vesicles

Under certain conditions, usually during periods of high nuclear activity, some cells exhibit vesiculation of one or both of the nuclear membranes (Frasca et al., 1965; Darlington and Moss, 1968, 1969; Krauskopf, 1968; Nii et al., 1968; Adams and Hertig, 1969; Killarski and Jasinski, 1970; Sylvester and Richardson, 1970; Gulyas, 1971; Franke and Scheer, 1974). Most of the vesicles that are formed are found on the cytoplasmic side of the nuclear envelope suggesting that if it is a method of transport it is more likely to be a route of nuclear exit rather than nuclear entry.

d) Transport during mitosis

During mitosis the nuclear envelope fragments and there is a generalised mixing of the cytoplasmic and nucleoplasmic components. This mixing has been demonstrated using both autoradiographic (Prescott and Goldstein, 1968) and immunofluorescent techniques (Beck, 1962; Gilden et al., 1965). Towards the end of mitosis the nuclear envelope begins to

reassemble and it is therefore conceivable that proteins too large to pass through nuclear pores could enter nuclei by becoming entrapped within the reforming nucleus. Such a route of nuclear entry has been demonstrated in the amoeba Chaos chaos (Feldherr, 1966), and these workers also found that immediately after mitosis the nuclei allowed the entry of particles which were too large to enter the nuclei during interphase. Whether these large particles entered the post-mitotic nuclei via gaps in the reforming nuclear envelope or whether their entry was due to differences in the nuclear pore structure between the post-mitotic and the interphase nuclei is not known. However, the fact that proteins larger than 70,000 daltons can enter interphase nuclei (Bonner, 1975a; De Robertis et al., 1978; Dingwall et al., 1982) suggests that, at best, specific entry at mitosis is a relatively minor route, although it could be important with regard to cell differentiation in those cases where differentiation is preceeded by cell division (see Dienstman and Holtzer, 1975).

e) Nucleus-associated synthesis of proteins

It is possible that some nuclear proteins are synthesised in association with the nucleus and this association could mean that they do not cross the nuclear envelope as such. One can suggest three ways in which this could happen. The first of these is the case where the proteins may be synthesised by the ribosomes found on the outer nuclear membrane and cross the envelope co-translationally in a nascent or unfolded state. Alternatively the proteins could be synthesised by the ribosomes on the

endoplasmic reticulum and be transported to the perinuclear space via the connecting pathways that exist between the ER and the nuclear envelope. Obviously these proteins would still need to cross the inner nuclear membrane, but one could suggest that they exist in the perinuclear space in an immature form that is capable of passing through the membrane. Both these possibilities arise from electron microscopy observations (Gorovsky, 1969; Kuehl and Sumsion, 1971), and there is, as yet, no direct evidence for them acting as pathways of nuclear entry.

Since translationally active polysomes have been prepared from a variety of cell nuclei (Goidl et al., 1975; Chatterjee et al., 1977; Gozes et al., 1977), it is possible that some proteins could be synthesised within the nucleoplasm. From studies on these nuclear polysomes, however, it has been estimated that the translational activity of the nucleus represents less than 1% of the total cell activity (Allen, 1978), and it is therefore unlikely to represent a major source of nuclear proteins. A possible role for this nuclear synthesis is suggested by the fact that some of the proteins synthesised by nuclear polysomes are apparently not produced by cytoplasmic polysomes (Gozes et al., 1977), and although these proteins have not been identified they could be regulators of nuclear activity.

As suggested above the nucleus-associated synthesis of proteins is unlikely to represent major routes of entry into the nucleus and this belief is reinforced by the ability of mature nuclear proteins to cross the nuclear envelope (see, for example, Bonner, 1975a; De Robertis et al., 1978; Dabauvalle and Franke, 1982) and the fact that most nuclear proteins are synthesised in the cytoplasm.

SECTION VI

THE ACCUMULATION OF PROTEINS IN NUCLEI

Whereas the previous section dealt with the passage of proteins across the nuclear envelope this section is concerned with the selective accumulation of some of these proteins in the nucleus.

Almost any substance that can pass easily across the nuclear envelope will accumulate to some extent in the nucleus *in vivo* (for examples see Bonner, 1978). Whilst the phenomenon of cytoplasmic exclusion can explain low level accumulation (see 'Methods A'), many proteins have been found to accumulate to very high levels in the nuclei of a variety of cells including *Xenopus* oocytes (Gurdon, 1970; Bonner, 1975a, 1975b; De Robertis *et al.*, 1978; Mills *et al.*, 1980; Dabauvalle and Franke, 1982; Dingwall *et al.*, 1982), amoebae (Legname and Goldstein, 1972; Jelinek and Goldstein, 1973), rat uterine cells (Stumpf, 1968) and giant salivary gland cells (Kroeger *et al.*, 1963). Two alternative models are commonly considered to explain the nuclear accumulation of these proteins (Dingwall *et al.*, 1982; De Robertis, 1983; Hall *et al.*, 1984). The first suggests that the accumulation of nuclear proteins is due to their selective transport across the nuclear envelope while the second envisages the free diffusion of all proteins into the nucleus with the subsequent retention of nuclear proteins by their binding to non-diffusible nuclear elements. The relative merits of these models are discussed below.

1. Selective transport across the nuclear envelope

This section does not refer to the mediated transport of large nuclear proteins since although specific import mechanisms have been inferred for such proteins (see, for example, Feldherr et al., 1983) there is no evidence that these are the cause of nuclear accumulation.

The classical way to test the function of a particular component in a system is to perturb or remove that component and then assess the effect of such a manoeuvre on the system. Both types of experiment have been performed on the nuclear envelope. It is possible to damage the nuclear envelope in Xenopus oocytes by repeatedly puncturing it with a glass needle (Feldherr and Pomerantz, 1978; Feldherr and Ogburn, 1980). Such action dramatically increases the rate at which large non-nuclear proteins are transported into the nucleus but has no effect on the nuclear uptake of nuclear proteins. Secondly, De Robertis and co-workers have succeeded in removing the nuclear envelope from isolated Xenopus oocyte nuclei and injecting the resulting nuclear gel into the cytoplasmic region of fresh oocytes. The re-introduced "nuclei" were still capable of accumulating nuclear proteins (cited in De Robertis, 1983). Although these results suggest that the nuclear envelope plays little or no role in the accumulation of most proteins in the nucleus, it is important in the nuclear accumulation of nucleoplasmin (Dingwall et al., 1982). Nucleoplasmin is the most abundant protein in oocyte nuclei (Krohne and Franke, 1980a; Mills et al., 1980) and accumulates in the nucleus following injection into the cytoplasm (Mills et al., 1980). In oocytes the nucleoplasmin exists as a pentamer of 168,000

daltons and protease digestion reveals the presence of two structural domains: a protease resistant core and five sensitive tail regions (Dingwall et al., 1982). By sequential removal of the tails it has been shown that the rate of nuclear accumulation, but not the final extent of accumulation, depends on the number of tails remaining. The complete removal of the tails leaves a core particle which remains in whichever cellular compartment it was micro-injected into, while the tails themselves accumulate in the nucleus (Dingwall et al., 1982). These results suggest that the nuclear accumulation of nucleoplasmin is due to selective transport across the nuclear envelope rather than selective retention within the nucleus and that the information for this accumulation resides in the protease sensitive tail region of the protein.

2. Selective binding within the nucleus

Any system involving the binding of one component to another will exhibit specificity, saturation and competition. All three of these properties are exhibited by the accumulation of histones in the nuclei of Xenopus oocytes (Gurdon, 1970; Bonner, 1975a). The specificity of the accumulation is obvious since histones injected into the cytoplasm of oocytes become more than 100-fold more concentrated in the nucleus than in the cytoplasm within 20 h (Gurdon, 1970). The saturation and competition aspects were demonstrated in a similar study using purified histone fractions (Bonner, 1975a). These properties are also evident in the nuclear accumulation of other nuclear proteins in Xenopus oocytes

(Bonner, 1975b), hormone receptors (Stumpf, 1968; Williams and Gorski, 1972), and the shuttling proteins in amoebae (Goldstein and Ko, 1974). Another characteristic of the nuclear accumulation of a variety of proteins is that their binding within the nucleus is weak. Binding constants calculated for histones and hormone receptors, for example, are about 10^{-4} to 10^{-5} M (Williams and Gorski, 1972; Bonner, 1978). It therefore seems that the nuclear accumulation of many proteins may be explained by their passive transport into the nucleus and their subsequent binding to non-diffusible intranuclear structures. If this is so it poses the problem of identifying these intra-nuclear structures.

Although hormone receptors appear to bind to DNA in the nucleus (Yamamoto and Alberts, 1976; Bonner, 1978) the situation with other nuclear proteins is less clear. On purely logical grounds it is unlikely that histones are accumulated by a similar binding to DNA. Under normal conditions Xenopus oocytes contain about 50 pg of DNA with one of each histone molecule bound every 25 base pairs, but following the injection of saturating amounts of purified H2B there is the equivalent of 32 H2B molecules per base pair of DNA (Bonner, 1975a). The fact that the different histone fractions accumulate to different extents is also suggestive that they are not accumulating as nucleohistone (Bonner, 1975a). A possible insight into this accumulation of histone is the finding that, in oocyte nuclei, histones H3 and H4 are bound to two related acidic nuclear-migrating proteins called N1 and N2 (Kleinschmidt and Franke, 1982).

These findings have led to the proposal that the nuclear binding sites are quasi-functional, that is they do not represent the sites at which the proteins ultimately function (see Bonner, 1978). This is almost certainly true for hormone receptors and histones, but it is not possible to comment on the majority of nuclear proteins since their functions remain unknown. A possible candidate for a quasi-functional binding component, at least in Xenopus oocytes, is nucleoplasmin. As mentioned previously, nucleoplasmin is the most abundant protein in the oocyte nucleus and such a role would not only explain its vast abundance but could also be a reason for the relatively unusual uptake mechanism thought to be employed by this protein (Dingwall et al., 1982). It could be, however, that there is no binding component as such but that nuclear accumulation is the result of complex interactions between several molecules which are either the same or different. It is known, for example, that the protein concentration within cells is very high and under these conditions protein-protein interactions can become much more important than in dilute aqueous solutions (for discussion see Fulton, 1982).

This second model proposes that all proteins enter nuclei by passive diffusion through nuclear pores. In Xenopus oocyte nuclei these pores have a permeant radius of 45 Å (Paine et al., 1975), and if transport through the pore was by passive diffusion then one could predict both the rate at which small proteins would enter these nuclei and also the maximum size of protein that should enter the nuclei. As discussed earlier, whereas non-nuclear proteins obey these rules after injection into Xenopus oocytes, nuclear proteins do not.

3. Karyophilic signals

Since nuclear accumulation is selective and occurs post-translationally with no apparent modification of the proteins (Gurdon, 1970; Bonner, 1975a, 1975b; De Robertis et al., 1978; De Robertis and Black, 1981; Dabauvalle and Franke, 1982; Dingwall et al., 1982), it has been proposed that nuclear proteins contain within their mature molecular structure a signal sequence which controls their accumulation in nuclei (De Robertis et al., 1982).

In recent years, two types of approach have been used to identify and localise such karyophilic signals in a variety of nuclear proteins. The first is exemplified by the work of Dingwall et al. (1982) who used partial proteolysis of nucleoplasmin to produce two fragments, only one of which retained the ability to migrate into and accumulate in the oocyte nucleus. This approach has also been used to locate the karyophilic signal of Xenopus histone H1 to the carboxyl-terminal domain of this protein (Dingwall and Allan, 1984). The second approach involves the use of recombinant DNA technology to construct genes in which various regions have been deleted or altered. In this way Kalderon et al. (1984) have identified a region of the SV40 large-T antigen necessary for its accumulation in the nuclei of transformed Rat-1 cells. A further modification of this approach involves the construction and expression of chimeric genes containing various regions of a nuclear protein fused to a cytosolic protein. In this way Hall et al. (1984) have identified an amino-terminal region of the yeast protein $\alpha 2$ which can cause the accumulation of bacterial β-galactosidase in the nuclei of yeast cells.

The work described in this thesis attempts to identify the karyophilic signal sequence in the nucleoprotein of influenza virus. As a host system we have used Xenopus laevis oocytes.

SECTION VII

INFLUENZA VIRUS

1. Morphology and polypeptides

There are three sub-types of influenza viruses, A, B and C, distinguished by the serological relatedness of their internal nucleoprotein antigen. They all have similar morphologies (Flewett and Apostolov, 1967), and can be either filamentous or spherical. Both forms have diameters of 80 to 120 nm and the filamentous forms are at least 4 μm in length. The percentage of a sample exibiting spherical morphology increases with the number of passages in culture (Choppin et al., 1960). Since only sub-type A viruses are used in the work described in this thesis the following discussion is limited to this type.

The virion has a lipid envelope that is derived from the host cell (Kates et al., 1961; Klenk and Choppin, 1969, 1970), and bears transmembrane surface protein projections or spikes. On the average spherical virion there are about 700-900 spikes packed in a honeycomb arrangement (Wrigley, 1979). These spikes are of two types: rod-shaped spikes of haemmaglutinin (HA) and, in much lesser abundance, mushroom-shaped spikes of neuraminidase (NA) (Laver and Valentine, 1969). On the inner surface of the lipid bilayer is an electron-dense layer composed of the viral membrane or matrix protein (M). This is believed to stabilise the structure of the particle, and may also act as an organiser for viral assembly at the plasma membrane. Internal to this layer are the ribonucleoprotein (RNP) structures which consist of four protein components and eight different segments of single stranded RNA. The predominant protein component is the nucleoprotein (NP) and the

three other proteins, PB1, PB2 and PA, are found in much smaller quantities. The RNA segments, which are of the opposite polarity to messenger RNA and therefore, by convention, termed negative sense (Baltimore, 1971), constitute the viral genome. Characterisation of the segmented genome and the assignment of specific segments to virus polypeptides is reviewed in Lamb (1983). The RNA segments are numbered according to their order of migration on glyoxal gels (Desselberger and Palese, 1978), and the sections below describe the polypeptide(s) encoded by each segment.

a) RNA segments 1-3 : The P proteins

The three largest RNA segments direct the synthesis of single protein products with molecular weights ranging from 82,000 to 95,000. These proteins, designated PB1, PB2 and PA (Horisberger, 1980), are components of the transcriptase complex found in both virus particles (Bishop et al., 1972) and infected cells (Caliguiri and Compans, 1974). Although all three are believed to be involved in RNA synthesis their individual roles remain unclear. From the use of temperature sensitive mutants PB1 and PB2 appear to be involved in mRNA synthesis while PA appears to play a role in the production of new viral genomes (Krug et al., 1975; Scholtissek and Bowles, 1975; Palese et al., 1977). In vitro transcription studies indicate that PB2 is responsible for binding the cap-structure of the host-cell primer mRNA (Ulmanen et al., 1981, 1983; Blaas et al., 1982; Penn et al., 1982) while PB1 is involved in initiating transcription (Ulmanen et al., 1983).

b) RNA segment 4 : The haemagglutinin

The haemagglutinin (HA) is an integral membrane protein with a molecular weight of 77,000. It is the major surface antigen of the virus and is the antigen against which neutralising antibodies are directed (Laver and Kilbourne, 1966). Changes in the antigenic structure of this molecule are associated with the recurrent epidemics observed with influenza. As well as its role in mediating the attachment of the virus to the plasma membrane of the susceptible host cell, it is also responsible for the entry of the virus into the cell (Klenk et al., 1975; Lazarowitz and Choppin, 1975).

The HA is synthesised as a single polypeptide chain and its biosynthetic route follows that taken by many cellular integral membrane proteins. This route is described in more detail elsewhere in the thesis and is therefore only briefly outlined here. At some time during its co-translational insertion into the endoplasmic reticulum the amino-terminal signal peptide is removed (Air, 1979; McCauley et al., 1979), and the nascent polypeptide undergoes core glycosylation (Compans, 1973a; Hay, 1974; Klenk et al., 1974; Elder et al., 1979; McCauley et al., 1980). Further glycosylation modifications occur during transport from the ER to the plasma membrane (Compans, 1973b; Rott and Klenk, 1977). In the majority of cases the HA is proteolytically cleaved into a heavy amino-terminal (HA1, MW ~ 50,000) and a light carboxyl-terminal (HA2, MW ~ 27,000) chain. This cleavage depends upon the virus strain, the host cell type and the growth conditions being studied (Lazarowitz et al., 1971, 1973a, b; Klenk et al., 1972;

Skehel, 1972). It does not affect the antigenic or receptor binding properties of the HA (Lazarowitz et al., 1971, 1973a, b; Lohmeyer and Klenk, 1979; McCauley et al., 1980) but does increase the infectivity of the virus (Klenk et al., 1975; Lazarowitz and Choppin, 1975) and hence is important for pathogenicity (Bosch et al., 1970; Rott, 1979) and the spread of infection in the organism (Rott et al., 1980). The two chains are not separate entities but remain bound by disulphide bridges as a single subunit (Laver, 1971). In the final virus particle three such subunits are non-covalently associated to form a single HA spike (Wiley et al., 1977).

c) RNA segment 5 : The nucleoprotein

The nucleoprotein (NP) is the type specific antigen of the influenza viruses although NPs from the same influenza type do show minor antigenic differences (Schild et al., 1979). It is the major protein that interacts with virus specific RNA segments of both polarities (Pons, 1971, 1975) to produce the viral ribonucleoproteins (RNPs) (Pons et al., 1969; Krug, 1971; Compans et al., 1972). The fact that the NP is a component of the transcriptase complex could indicate that, like the P proteins discussed above, it has an enzymic role in transcription. Indeed the addition of NP specific monoclonal antibodies to the transcriptase complex inhibits its activity in vitro (van Wyke et al., 1980), but whether this inhibition is due to steric hinderance or interference with a non-structural activity of NP is unclear. When temperature sensitive mutants with lesions in the NP gene are grown at

the non-permissive temperature they synthesize normal yields of virion RNA and other components but no infectious progeny are produced (Scholtissek and Bowles, 1975). This may be due to an inability of the RNPs to be packaged into progeny. It has been proposed that packaging involves an interaction of the RNPs with the matrix protein at the plasma membrane (Choppin et al., 1972; Compans and Choppin, 1975), as has been suggested for Sendai virus (Shimizu and Ishida, 1975). This idea is supported by biochemical evidence showing a specific association of matrix protein with RNPs (Rees and Dimmock, 1981a).

During infection a proportion of the NP molecules become phosphorylated (Privalskya and Penhoet, 1977, 1978). This phosphorylation occurs within ten minutes of the synthesis of the NP and as the infection progresses increasing amounts of the newly-synthesised molecules become phosphorylated (Almond and Felsenreich, 1982). In vitro results indicate that phosphorylation stimulates the activity of the transcriptase complex (Kamata and Watanabe, 1977) while in vivo work suggests that it is associated with the migration of the NP into the nucleus (Almond and Felsenreich, 1982). This later point is disputed by results obtained in our laboratory (Petri and Dimmock, 1981). Another possibility (for which there is no evidence) is that the phosphorylation alters the specificity of the transcriptase complex to accomodate the synthesis of template, messenger and virion RNA.

d) RNA segment 6 : The neuraminidase

Like the HA the neuraminidase (NA) is an integral membrane glycoprotein and it is therefore not surprising that they share many common biosynthetic steps. The NA is co-translationally inserted into the membrane of the ER where it is core glycosylated. Further glycosylation occurs during its transport to the plasma membrane (Compans, 1973a, b; Stanley et al., 1973; Hay, 1974; Klenk et al., 1974). Unlike the HA however, the signal sequence is not removed and is responsible for retaining the protein in the membrane (Fields et al., 1981; Blok et al., 1982). This means that the NA is orientated with its amino-terminus inserted into the viral membrane. Differences are also observed in the make up of the final viral spike which in the case of NA contains four uncleaved molecules of about 56,000 daltons bound by disulphide bounds (Lazdins et al., 1972).

The role(s) of the NA is not fully understood but all of its proposed functions employ its ability to remove terminal n-acetylneuraminic acid (sialic acid) residues from carbohydrate chains in glycoproteins (Gottschalk, 1957). The earliest proposal was that the NA mediates the release of the progeny virus from the host cell surface (Gottschalk, 1965; Seto and Rott, 1966; Webster and Laver, 1967; Compans et al., 1969). More recent work using fragments of monoclonal antibodies suggests that this is not correct (Becht et al., 1971). These fragments inhibit the enzymic acitivity of the NA but do not prevent the release of progeny virus.

Since influenza virions do not normally contain sialic acid residues, it has been suggested that the NA is responsible for removing these residues from the region of the plasma membrane where viral maturation is occurring (Klenk et al., 1970). Support for this suggestion comes from the use of a temperature sensitive mutant defective in NA activity (Palese et al., 1974). The progeny virions produced at the non-permissive temperatures were clumped into large aggregates since they contained sialic acid residues which served as receptors for the viral HA. This removal of the sialic acid residues is also thought to be necessary for the cleavage of the HA (Schulman and Palese, 1977), but there is contradictory evidence (Bosch et al., 1979).

Evidence for an involvement of the NA in the uptake of the influenza virus is similarly contradictory. The use of antibodies suggest that the NA is not involved in uptake (Seto and Rott, 1966; Webster and Laver, 1967; Kilbourne et al., 1968; Compans et al., 1969), whereas studies on the fusion of cell membranes with liposomes containing influenza glycoproteins reveal an absolute requirement of the NA for membrane fusion (Huang et al., 1980b). Both approaches are open to criticisms, for example, the antibodies may not completely inhibit NA activity while membrane fusion between the host cell and the influenza virus has not been demonstrated in vivo. An absolute requirement for the NA in membrane fusion is also difficult to reconcile with the fusion of CV-1 cells expressing a cloned HA gene (White et al., 1982).

In many animals influenza is an infection of the respiratory tract. The tract is lined with sialomucoproteins which seem to bind the viral HA

and thereby reduce infectivity (Shen and Ginsberg, 1968). The NA may free the virus from these proteins and therefore help the virus to reach its target cells.

e) RNA segment 7 : The matrix protein (M) and the non-structural protein M2

Three major mRNA species are transcribed from RNA segment 7 (see Lamb and Lai, 1981; Lamb et al., 1981). The most abundant is a colinear transcript which encodes the matrix protein (M). The other mRNAs are produced as a result of splicing of this transcript.

M has a molecular weight of about 25,000 and is the most abundant polypeptide in the virus particle (Skehel and Schild, 1971). It seems to form a protein shell beneath the virion envelope (Apostolov and Flewett, 1969; Compans and Dimmock, 1969) which may give structural support to the virion (Laver, 1973; Schulze, 1973). The fact that virions produced at 39°C have half the normal M content and decreased stability (Kendal et al., 1977a) supports this idea of a structural role. The ability of M to interact with both lipid (Bucher et al., 1980; Gregoriades, 1980; Gregoriades and Frangione, 1981) and viral RNPs (Reginster and Nermut, 1976; Rees and Dimmock, 1981a) could indicate a role for M in viral assembly (see Choppin et al., 1972 and Compans and Choppin, 1975). Such a role for the corresponding M protein has been suggested in the assembly of Sendai virus particles (Shimizu and Ishida, 1975).

The major spliced transcript of segment 7 encodes the non-structural protein M2. M and M2 share the same nine amino acids at their amino termini but the splicing process means that the remainder of the M2 mRNA is translated in the +1 reading frame (Lamb et al., 1981). From its nucleotide sequence M2 has a molecular weight of about 11,000 (Lamb et al., 1981), but it migrates anomalously on polyacrylamide gels with a molecular weight of about 15,000 (Lamb and Choppin, 1981). It is only found in infected cells (Lai et al., 1980; Lamb and Choppin, 1981) and no information is yet available regarding its function. A second interrupted mRNA is also transcribed from RNA segment 7 (Inglis and Brown, 1981; Lamb et al., 1981). This mRNA, called M3 mRNA, has the coding potential for a peptide of nine amino acids which would be the same as the last nine amino acids of M, but there is no evidence for the existence of such a peptide in either infected cells or virus particles.

RNA segment 7 has been implicated in the differential susceptibility of influenza virus strains to the antiviral drug amantidine (Lubeck et al., 1978; Hay et al., 1979). It has not been possible however to determine either the mechanism of inhibition or the mechanism of resistance.

f) RNA segment 8 : The non-structural proteins NS1 and NS2

As with RNA segment 7, segment 8 directs the synthesis of more than one transcript. The larger, colinear transcript is not only translated to give NS1 but is also spliced to give the NS2 mRNA (for discussion see Lamb, 1983). NS1 and NS2 share the same ten amino acids at their amino

termini but the splicing process means that the remainder of the NS2 mRNA is translated in the +1 reading frame (Lamb and Lai, 1980). Neither NS1 nor NS2 are found in virus particles.

NS1 has a predicted molecular weight of about 26,000 but runs on gels with an apparent molecular weight of only 23,000. It is synthesised in large amounts early in infection (Lazarowitz et al., 1971; Skehel, 1972; Meier-Ewert and Compans, 1974) and has been found associated with polysomes and nucleoli (Lazarowitz et al., 1971; Pons, 1972; Compans, 1973a; Krug and Etkind, 1973; Krug and Soiero, 1975). It has been implicated in the synthesis of viral RNA (Almond et al., 1977; Maeno et al., 1979; Wolstenholme et al., 1980) and the inhibition of host protein production (Stephenson and Dimmock, 1974; Krug and Soiero, 1975). Late in infection it is often involved in the formation of cytoplasmic paracrystalline inclusions (Morrongiello and Dales, 1977; Shaw and Compans, 1978) and the purification of these inclusions show them to be composed of NS1 in association with RNA that is primarily cellular in origin (Yoshida et al., 1981). Although these inclusions may have a functional significance it is more likely that they arise as a consequency of the great abundance of NS1 in the dying cell and because its basic nature attracts RNA.

From its nucleotide sequence NS2 has a predicted molecular weight of 14,000 but runs on gels with an apparent molecular weight of 11,000. It is synthesised late in infection and is predominantly cytoplasmic (Skehel, 1972; Follett et al., 1974; Minor and Dimmock, 1975; Lamb and Choppin, 1978, 1979; Lamb et al., 1978). No function has yet been assigned to this protein.

2. The association of influenza virus proteins with the nuclei of infected cells

The replication of influenza virus has an absolute requirement for an active host cell nucleus (Follett et al., 1974; Kelly et al., 1974). The principal nuclear function that is required is the synthesis of host cell mRNA which serves as a primer for the transcription of virus-specific mRNA (for discussion see Krug et al., 1981). It has also been suggested that the synthesis of viral RNA (vRNA) (Avery, 1974; Barrett et al., 1979; Barry and Mahy, 1979; Mark et al., 1979; Krug et al., 1981; Jackson et al., 1982) and the initial association of this RNA with virus proteins prior to their incorporation into progeny virions (Flawith, 1979) takes place in the nucleus. Not surprisingly therefore, the majority of the influenza proteins can be found in the nucleus at some stage during the replication cycle.

a) The P proteins

Studies on the localisation of the P proteins is difficult since they are synthesised in low amounts throughout infection. Early work was inconclusive since although the P proteins could be detected in the nuclei of infected cells the gel systems used in these studies were unable to resolve the three P proteins (Krug and Etkind, 1973; Hay and Skehel, 1975), and it was therefore possible that the proteins behaved differently to each other. Indeed more recent studies, using improved gel systems, have shown that early in infection PB1 and PB2 accumulate

in the nucleus to a greater extent than PA, but by 6 h p.i. all three proteins are accumulated in the nucleus to the same extent (Cook et al., 1979b; Briedis et al., 1981). The preferential accumulation of PB1 and PB2 in the nucleus early in infection is in agreement with their proposed roles in mRNA synthesis and the belief that mRNA synthesis occurs in the nucleus. Similarly the increased nuclear migration of PA later in infection agrees with its proposed involvement in vRNA synthesis and with the suggestion that this synthesis also occurs in the nucleus.

b) The nucleoprotein

NP was first detected in the nuclei of infected cells by specific immunofluorescence staining (Watson and Coons, 1954; Liu, 1955; Breitenfeld and Schäfer, 1957), and the analysis of radiolabelled proteins following the fractionation of infected cells revealed that after its synthesis in the cytoplasm the majority of the NP rapidly migrates to the nucleus and associates with the nucleoplasm (Dimmock, 1969; Joss et al., 1969; Taylor et al., 1969, 1970; Lazarowitz et al., 1971; Krug and Etkind, 1973; Hay and Skehel, 1975; Briedis et al., 1981). This nuclear accumulation and the fact that most of the NP in the nucleoplasm is in the form of ribonucleoprotein complexes (Krug, 1972; Krug and Etkind, 1973) is consistent with its predicted role as a component of the viral transcriptase. The observation of a 45 min lag between the synthesis of NP and its incorporation into progeny virions (Hay and Skehel, 1975) could also mean that the NP must enter the

nucleus prior to this incorporation. Studies employing both immunofluorescence staining (Breitenfeld and Schäfer, 1957; Fraser, 1967; Maeno and Kilbourne, 1970) and radioactive pulse-labelling (Flawith and Dimmock, 1979), do indeed suggest that the NP migrates back out of the nucleus and the time of peak virion release fits chronologically with the notion that nuclear NP becomes virion NP (Flawith and Dimmock, 1979). These last results should be viewed with caution since it is not possible to distinguish between those protein molecules which have remained in the cytoplasm and those which have migrated into the nucleus and then been transported out again.

c) The matrix protein and M2

Within a few minutes of its synthesis the matrix protein is found associated with all of the cellular membranes (Klenk et al., 1974; Meier-Ewert and Compans, 1974; Hay and Skehel, 1975), but its migration to the nucleus remains debatable. Some investigators claim that M becomes accumulated in the nucleus (Gregoriades, 1973, 1977; Hay and Skehel, 1975; Flawith and Dimmock, 1979), while others claim it is entirely cytoplasmic (Lazarowitz et al., 1971; Krug and Etkind, 1973; Mahy et al., 1980; Briedis et al., 1981). The likely explanation is that the nuclear accumulation of M depends upon the virus strain and/or the host cell type being studied. No information is yet available on the cellular location of M2.

d) The non-structural proteins NS1 and NS2

Following its synthesis in the cytoplasm a proportion of the NS1 migrates to the nucleus (Taylor et al., 1969, 1970; Lazarowitz et al., 1971; Hay and Skehel, 1975) and accumulates in the nucleoli (Dimmock, 1969; Krug and Etkind, 1973; Krug and Soiero, 1975). Its association with nucleoli could be a result of its ability to bind RNA or may be connected with its proposed inhibition of host protein synthesis. NS2 appears to remain predominantly in the cytoplasm (Briedis et al., 1981) and an earlier report claiming a nuclear location (Krug and Etkind, 1973) may have been confused by the presence of fragments of NS1 (Lamb et al., 1978).

e) The viral glycoproteins

The two viral glycoproteins, the haemagglutinin and the neuraminidase, do not enter the nucleus during infection (Breitenfeld and Schäfer, 1957; Dimmock, 1969; Maeno and Kilbourne, 1970; Oxford and Schild, 1975). This is not surprising since following their synthesis on the rough endoplasmic reticulum they are transported to the plasma membrane via smooth internal membranes (Compans, 1973a; Stanley et al., 1973; Hay, 1974; Klenk et al., 1974).

SECTION VIII

XENOPUS LAEVIS OOCYTES

Fully grown Xenopus laevis oocytes have a diameter in excess of 1 mm and contain a reserve of various macromolecules and organelles which are used during early embryonic development (Woodland, 1982). These features have made oocytes attractive hosts for the introduction of a variety of macromolecules such as proteins, RNA and DNA (see below) and organelles (Richter and Smith, 1981). The principal advantage over the simpler and usually more economical cell-free systems is the ability of the oocyte to carry out the modification, processing and segregation of many foreign proteins. In the last few years numerous exhaustive reviews extolling these properties have been published (Asselbergs, 1979; Gurdon and Melton, 1981; Wickens and Laskey, 1981; Lane, 1983; Colman et al., 1984; Soreq, 1984) and rather than repeat the findings at length I will summarise the properties which make the oocyte well suited for use as a surrogate host in many situations. References are only included where specific examples are quoted and the reader is referred to the above reviews for further references.

1. Cleavage of polypeptides

As discussed elsewhere, the majority of secretory and integral membrane proteins are synthesised with an amino-terminal signal sequence which specifies their association with cellular membranes and is removed during, or soon after, translocation. The first indication that oocytes could correctly process such proteins came from studies involving the injection of mRNAs for secretory and non-secretory proteins. Secretory proteins were incorporated into membrane vesicles (Zehavi-Willner and

Lane, 1977) and subsequently exported (Colman and Morser, 1979), while non-secretory proteins remained in the cytosol. In several cases the mobility of the vesicular form was greater than the *in vitro* product (see Colman *et al.*, 1984) suggesting the correct removal of the signal peptide although a direct demonstration, using amino-terminal amino acid sequencing, has only been made in one case (Lane *et al.*, 1981a).

Many proteins exist in the lumen of the endoplasmic reticulum as a propeptide with a further amino-terminal peptide which is usually removed intracellularly during maturation. Oocytes are unable to perform this second cleavage with promellitin (Lane *et al.*, 1981a) and carp pro-insulin (Rapoport, 1981) suggesting that the cleavage enzymes are probably tissue specific. Frog vitellogenin, on the other hand, is cleaved to phosvitin and lipovitellin in yolk platelets following its secretion from, and endocytosis by, oocytes which have been previously injected with mRNA (Lane *et al.*, 1983). The correct cleavage of a number of viral proteins has also been observed in oocytes (for references see Lane, 1983).

2. Glycosylation

Most, if not all, secretory and membrane proteins are glycosylated. The initial step is usually the *en bloc* addition of oligosaccharide chains to selected asparagine residues in the nascent peptide (core glycosylation). These side chains are subsequently modified during the transport of the protein through the cell (peripheral glycosylation)

(for review, see Hubbard and Ivatt, 1981). Oocytes add oligosaccharide chains to only those proteins which, in their "native" state, are N-glycosylated (Jilka et al., 1979). Since the composition of these "core" chains is tissue specific it is probable, but not yet proven, that the chain added by the oocyte will differ from the chains in the "native" proteins. It is known however that, in some cases at least, the subsequent modifications in oocytes are different from those in the "native" cell (Mous et al., 1980, 1982).

3. Phosphorylation

Oocytes are able to phosphorylate the translation products of several injected mRNAs encoding phosphoproteins. These include trout testis protamine (Gedame et al., 1978) and frog vitellogenin (Berridge and Lane, 1976) which is phosphorylated prior to cleavage. Synthetic peptides injected into oocytes are also phosphorylated (Maller et al., 1978). Guinea-pig caseins produced in oocytes however, are not phosphorylated (Lane, 1983) suggesting that at least some phosphorylation events are tissue specific.

4. Post-translational modification of amino acid residues

The post-translational modification of many amino acid residues occurs correctly in oocytes. These modifications include the acetylation of amino-terminal methionines in calf lens A2-crystallin (Berns et al.,

1972), the amino acetylation of sea urchin histones (Woodland and Wilt, 1980) and the hydroxylation of proline residues in mouse fibroblast collagen (Lane and Knowland, 1975). Certain types of modification such as the iodination of thyroglobulin (Vassart et al., 1977) and the synthesis of the C-terminal amide of promellitin (Kindas-Mugge et al., 1974) do not occur in oocytes. These modifications are probably tissue specific.

5. Assembly of multi-subunit proteins

In many cases the injection of mRNA into oocytes results in the non-covalent assembly of the products into an active protein complex. Probably the best example of this is the injection of torpedo electric organ mRNA which results in the synthesis and assembly of all four subunits of the nicotinic acetylcholine receptor into a functional receptor in the plasma membrane (Sumikawa et al., 1981; Miledi et al., 1982). Functional activity however should not be taken as evidence that the complex is identical to its "native" counterpart. Oocyte made mouse β-glucuronidase, for example, is active but not "native" (Labarca and Paigen, 1977). Not all oligomeric complexes are assembled correctly in oocytes (for examples see Asselbergs et al., 1978).

Covalent assembly via disulphide bridges has also been observed with oocytes (for examples see Deacon and Erbinger, 1979; Valle et al., 1981, 1982; Colman et al., 1982).

6. Segregation of proteins

The ability of oocytes to secrete a variety of proteins has been mentioned above. The only documented exception is bee venom promellitin. Although the signal peptide is removed from this protein it is not secreted to any significant extent (Lane et al., 1981a). This may be due to the inability of the oocytes to correctly cleave the propeptide.

The transport of proteins into nuclei (Lane et al., 1981b), yolk platelets (Berridge and Lane, 1976; Lane et al., 1983), intracellular membranes (Ohlsson et al., 1981) and storage granules (Hurkman et al., 1979, 1981; Larkins et al., 1980) have also been observed in mRNA-injected oocytes.

7. Conclusion

Nearly all of the examples cited above involve the injection of mRNA into oocytes. However, the oocyte can also be used as a coupled transcription-translation system as first demonstrated by De Robertis and Mertz (1977) and reviewed by Gurdon and Melton (1981), Wickens and Laskey (1981) and Lane (1983). The production of a functional eukaryotic mRNA normally involves the addition of a 5'-terminal "cap" and a 3'-terminal poly(A) tail and the removal of intervening sequences (for a review see Darnell, 1979). Although these processes can occur correctly in oocytes, as evidenced in the production of ovalbumin

(Wickens et al., 1980) and certain viral proteins (De Robertis and Mertz, 1978; Laskey et al., 1978; Rungger and Turler, 178; Rungger et al., 1979; McKnight and Gavis, 1980; Cordingley and Preston, 1981; Preston and Cordingley, 1982) it is quite inefficient when heterologous genes are used. In addition many eukaryotic promotors are either not used or used very inefficiently in Xenopus oocytes. A better choice for injection is a full length cDNA coupled to a powerful promotor as exemplified by the work of Krieg et al. (1984) who studied the synthesis and segregation of a variety of secretory proteins using plasmids containing cDNAs under the transcriptional control of the herpes simplex virus thymidine kinase promotor. In this way, these workers were able to identify those features of ovalbumin which are important to its segregation in membranes (Tabe et al., 1984). The potential use of oocytes for a similar study of the transport of nuclear proteins is even greater since the nucleus can be isolated manually and there is no suitable cell-free system for such studies.

SPECIFIC AIM

The specific aim of this work was to identify the information controlling the accumulation of the influenza virus nucleoprotein in the nuclei of *Xenopus* oocytes.

MATERIALS

1. Anaesthetics

Sodium Barbitone (Euthetal) was obtained from May and Baker Ltd., Dagenham, Essex. Ethyl m-amino-benzoate (MS222) was obtained from Sigma Chemical Co. Ltd., Poole, Dorset.

2. Antisera

FITC-conjugated goat-anti-rabbit immunoglobulin antiserum and rabbit anti-human globin antiserum were obtained from Miles Laboratories Ltd., Stoke Poges, Slough.

Mouse monoclonal antisera against the influenza virus NP were a kind gift from Dr. R. G. Webster (St. Jude Children's Research Hospital, Memphis, USA), (van Wyke et al., 1980).

Rabbit polyclonal antiserum against the influenza virus NP was a kind gift from Mr. A. S. Carver (University of Warwick), and was prepared by the subcutaneous injection of polyacrylamide gel-purified NP into half-lop rabbits (Hylyne, Marston, Cheshire).

3. Bacterial culture media

Antibiotic medium 3 (Bacto-Penassay Broth), Bacto-Agar, Bacto-Tryptone and casamino acids were obtained from Difco Laboratories, East Molesey, Sussex. Oxoid yeast extract was obtained from Oxoid Ltd., Basingstoke, Hampshire. Ampicillin and chloroamphenicol were obtained from Sigma Chemical Co. Ltd., Poole, Dorset.

4. Chemicals

All chemicals not listed were obtained as AnalaR grade from BDH Chemicals Ltd., Poole, Dorset. Dithiothreitol, ethidium bromide, HEPES, L-methionine, nucleotide triphosphates and Trizma were obtained from Sigma Chemical Co. Ltd., Poole, Dorset.

5. Electrophoresis components

Acrylamide and N-N'-methylene bisacrylamide (specially pure) were obtained from BDH Chemicals Ltd., Poole, Dorset. N,N,N',N'-tetramethylenediamine (TEMED) was obtained from BioRad Laboratories Ltd., Watford, Herts.

Agarose (Type II) was obtained from Sigma Chemical Co. Ltd., Poole, Dorset.

6. Enzymes

Calf intestinal phosphatase, Escherichia coli DNA polymerase (Klenow fragment) and T4 DNA ligase were obtained from Boehringer Corporation (London) Ltd., Lewes, Sussex. Nuclease Bal-31 and the restriction enzymes Aha II, Bgl I, Bgl II, Nae I, Nco I and Stu I were obtained from C.P. Laboratories, Herts. The restriction enzymes Acc I, Bam Hl, Cla I, Hae III, Hind III, Pvu II, Sal I and Sma I were obtained from Bethesda Research Laboratories Ltd., Cambridge. Lysozyme and ribonuclease A were obtained from Sigma Chemical Co. Ltd., Poole, Dorset.

7. Filter paper

Whatmann 3 MM filter paper was obtained from Whatman Sales Ltd., Maidstone, Kent.

8. Photographic components

Fuji RX X-ray film was obtained from Fujimex Ltd., Swindon. X-Omat S X-ray film was obtained from Kodak (Photographic) Ltd., Hemel Hempstead, Herts. DX 80 X-ray film developer and FX 40 X-ray film fixer were obtained from Kodak Chemicals Ltd., Kirkby, Liverpool. The Polaroid Land camera and Polaroid Land film type 665 were obtained from Polaroid (UK) Ltd., St. Albans, Herts.

9. Radiolabelled components

L-[^{35}S]-methionine, 1200-1400 Ci/mmol was obtained from Amersham International plc, Amersham, Bucks.

10. Tissue culture media

All tissue culture media and newborn calf serum were obtained from Flow Laboratories Ltd., Irvine, Ayrshire. The antibiotics benzyl penicillin (sodium) BP and streptomycin sulphate were obtained from Vestric Ltd., Brierley Hill, Staffs.

METHODS A

1. Cells

Primary chick embryo fibroblast (CEF) cells were prepared as described by Morser et al. (1973) and seeded at concentrations of 9×10^6 cells/3 ml in 5 cm plastic Petri dishes or 2×10^8 cells/200 ml in roller bottles. The monolayers were used when confluent.

Madin-Darby canine kidney (MDCK) cells (Flow Laboratories, Irvine, Ayrshire) were maintained as a continuous cell culture using Dulbecco's modified Eagle's medium containing 5% newborn calf serum.

Baby hamster kidney (BHK-21) cells (Flow Laboratories, Irvine, Ayrshire) were maintained as a continuous cell culture using Glasgow's modified Eagle's medium (GMEM) containing 5% newborn calf serum.

2. Viruses

The type A influenza viruses used in this study were the avian strain A/FPV/Rostock/34 (FP/R) (H7N1) and the human strain A/NT/60/68 (H3N2) (a kind gift from Dr. G. Both, CSIRO, Genetic Research Laboratory, New South Wales, Australia). Both were grown by inoculation of about 10^5 pfu into the allantoic cavity of 11-day embryonated chicken's eggs. Those infected with FP/R were incubated for 18 h at 37°C, and those infected with A/NT/60/68 for 48 h at 33°C. After chilling the eggs at 4°C overnight the allantoic fluid was collected, clarified by low speed centrifugation and snap-frozen in dry ice/ethanol before storing in 1 ml

aliquots at -70°C. This was used as inoculum.

The infectivity of FP/R was determined by plaque assay on monolayers of primary CEF cells as described by Dimmock and Watson (1969) and Meier-Ewert and Dimmock (1970).

The infectivity of A/NT/60/68 was determined by plaque assay on monolayers of MDCK cells under an agar/medium 199 overlay containing 0.04% DEAE-dextran, 0.14% BSA and 2.5 µg/ml trypsin-TPCK which cleaves the viral HA and renders progeny virus infectious (Appleyard and Maber, 1974; Klenk et al., 1975; Lazarowitz and Choppin, 1975). Because human strains are inhibited by serum, the cells were washed thoroughly in PBS and incubated with serum-free medium for 1 h prior to infection. The overlay also contained no serum.

3. Measurement of influenza virus haemagglutinin

Haemagglutinin was measured by making doubling dilutions in PBS and adding chicken erythrocytes to 0.5% (Borland and Mahy, 1968). The titre was determined by estimating the 50% end-point between full agglutination and no agglutination after 30 min at room temperature.

4. Radiolabelling and purification of FP/R virus

[^{35}S]-methionine-labelled FP/R for use as markers in polyacrylamide gel electrophoresis, was prepared in roller bottles containing monolayers of primary CEF cells. Cell monolayers were washed with prewarmed PBS and incubated in GMEM containing 1% newborn calf serum and 1/10th the normal methionine concentration (that is, 1.5 mg/litre) for 24 h before infecting with virus at a multiplicity of infection of 1 pfu/cell. After 1 h the inoculum was replaced with 50 ml of the above medium containing 1 mCi [^{35}S]-methionine. Incubation was continued for 20 h. Radiolabelled virus was purified essentially as described by Dimmock et al. (1977) except that no unlabelled carrier virus was added. Briefly, the tissue culture fluid was clarified by centrifugation at 1,000 g for 10 min at 4°C and the virus was precipitated with 60% saturated ammonium sulphate on ice. After centrifugation at 20,000 g for 20 min the virus pellet was resuspended in 3 ml of PBS and loaded onto a 60 ml 15-45% (w/v) linear sucrose gradient. Following centrifugation at 90,000 g for 90 min at 4°C the gradient was fractionated into 2 ml aliquots and assayed for haemagglutinin activity and radioactivity. Where these activities formed a coincident peak the fractions were pooled. The virus was pelleted by centrifugation at 75,000 g for 2 h at 4°C, resuspended in 10 mM Tris-HCl (pH 7.4) and stored in aliquots at -70°C.

5. Preparation of radiolabelled cell lysate

FP/R in allantoic fluid was added to monolayers of primary CEF cells in 5 cm Petri dishes to give a multiplicity of infection of 30 pfu/cell. After 1 h incubation at 37°C the inoculum was replaced with 2 ml of prewarmed GMEM containing 1/10th of the normal methionine concentration (that is, 1.5 mg/litre). 10 min prior to radiolabelling the cells were washed with prewarmed buffered Earle's saline (BES) and incubated with 2 ml of BES. The cell monolayers were now incubated in a metal tray floating on a water bath at 37°C. At the time of labelling (3.5 h after addition of virus) the medium was replaced with 0.2 ml of BES containing 50 µCi [^{35}S]-methionine, and after 10 min the cells were washed twice with ice cold resuspension buffer (RB) (8.8 mM NaCl, 1 mM HEPES, pH 7.6) and scraped into 0.1 ml of RB. The cells were disrupted with ultrasound at 1.5 A (Soniprobe, Dawe Instruments Ltd.), dialysed against RB, freeze dried and resuspended in distilled water to one-tenth original volume. Prior to injection into oocytes the lysate was centrifuged at 12,000 g for 3 min at 4°C to remove any particulate matter.

6. Preparation of mRNA

The solutions used in the preparation of mRNA are described in Table 1.

FP/R in allantoic fluid was added to monolayers of primary CEF cells in roller bottles (approximately 2 x 10^8 cells/bottle) to give a multiplicity of infection of 30 pfu/cell. After 1 h incubation at 37°C the inoculum was replaced with prewarmed medium 199 and incubation continued for a further 2.5 h. The cells were washed twice with ice cold PBS and scraped into 10 ml of the same solution using a rubber policeman. The cells were collected by centrifugation at 2,000 g for 5 min at 4°C and gently resuspended in 10 ml of ice cold homogenisation buffer (see Table 1). After homogenisation the nuclei were pelleted by centrifugation at 2,000 g for 5 min. The supernatant was collected, made to 1% SDS, 2 mM EDTA (using stock solutions) and left at room temperature for 15 min. After being extracted twice with phenol:chloroform (Table 3) and twice with chloroform, the RNA was precipitated with ethanol and dissolved in 4 ml of binding buffer (Table 1). The preparation was enriched for poly A^+ RNA by binding to oligo(dT)-cellulose and eluting with the same buffer lacking the LiCl. The polyadenylated RNA was ethanol precipitated, resuspended in distilled water to a concentration of 1 mg/ml and stored in aliquots at -20°C.

Table 1 Solutions for the preparation of mRNA

Homogenisation Buffer

0.2 M	Tris-acetate (pH 8.5)
0.35 M	Sucrose (ribonuclease free)
50 mM	KCl
10 mM	Magnesium acetate
1.3%	Triton X-100

Binding Buffer

10 mM	Tris-HCl (pH 7.5)
1 mM	EDTA
0.1%	SDS
0.5 M	LiCl

The solutions were prepared from autoclaved stock solutions. All glassware was heat-treated at 200°C for 6 h to inactivate ribonucleases.

7. DNA techniques

The Escherichia coli strain HB101 was used throughout the study. The bacteria were stored at -20°C in 15% glycerol.

All enzymes were used as recommended by their manufacturers and so, for convenience, the conditions are omitted. Additional information is outlined below.

a) Partial digestion of DNA

In many cases it was necessary to digest DNA at some, but not all, of the sites available to a particular restriction enzyme. To obtain the desired extent of digestion a reaction mixture was set up which would attain complete digestion after 90 min incubation. At various times during the incubation, samples were removed from the reaction and made to 1% SDS, 15 mM EDTA to stop digestion. From analysis by agarose gel electrophoresis it was possible to determine the time at which the desired extent of digestion had been obtained. An identical reaction mixture was then set up and allowed to incubate for this length of time before being made to 1% SDS, 15 mM EDTA. The desired fragment(s) was then purified as described in Section 12 of 'Methods A'.

b) Digestion of DNA with two restriction enzymes

When DNA was to be digested with two restriction enzymes employing the same buffer, the digestions were carried out simultaneously in a single reaction mixture. If, however, the enzymes used different buffers then the DNA was digested with the first enzyme, phenol:chloroform extracted

and ethanol precipitated and then digested with the second enzyme.

c) Nuclease Bal-31 digestion of DNA

Nuclease Bal-31 has an exonuclease activity which simultaneously degrades both the 3' and 5' termini of duplex DNA. To determine the length of time required to remove the desired number of nucleotides from a DNA sample a reaction mixture was set up as described by the enzyme manufacturers. At appropriate times, samples were removed from the reaction and made to 20 mM EGTA to stop digestion. From analysis by agarose gel electrophoresis it was possible to determine the time at which the desired amount of digestion had been obtained. An identical reaction mixture was then set up and allowed to incubate for this length of time before being made to 20 mM EGTA. The desired fragment was then purified as described in Section 12 of 'Methods A'.

8. Transformation of bacteria

50 ml of L-broth (see Table 2) in a 500 ml flask was inoculated with 1 ml of a 10 ml overnight bacterial culture. The cells were incubated at 37°C with vigorous shaking until the culture had reached 0.35 OD_{600} (typically 95 min). This was found to give approximately 10^7 viable cells/ml. After chilling the culture on ice for 40 min the cells were pelleted by centrifugation at 4,000 g for 5 min at 4°C, washed once in 25 ml of ice cold 0.1 M $MgCl_2$ and resuspended in 2.5 ml of ice cold 0.1 M $CaCl_2$ (freshly made). 0.2 ml aliquots were dispensed into pre-chilled tubes and stored at 4°C for 6 h. Between 10 and 40 ng of

Table 2 Preparation of bacterial media

L-Broth

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

Made to 600 ml with distilled water, titrated to pH 7.5 with 1 M NaOH and adjusted to 1 litre with distilled water before autoclaving.

For preparing plates Bacto-agar was added to a concentration of 1.5% (w/v) just prior to autoclaving.

When required, ampicillin was added to the cooled medium to a concentration of 100 µg/ml. When preparing plates containing ampicillin the autoclaved agar was allowed to cool to 45°C before addition of the ampicillin and pouring of the plates. A 25 mg/ml stock solution of the sodium salt of ampicillin in water was filter sterilised and stored in aliquots at -20°C.

ligated DNA in 0.1 M Tris-HCl (pH 7.4) was added to each tube, inverted and left on ice for 30 min with periodic inversions. The cells were heat shocked at 42°C for 2 min in a preheated water bath and returned to the ice bath for a further 30 min. To allow the bacteria to become resistant to ampicillin, 0.5 ml of L-broth was added to each tube and incubated at 37°C for 40 min. Ampicillin-containing plates (see Table 2) were spread with 0.2 ml aliquots of the transformation mixture and incubated inverted at 37°C for 16-20 h.

9. Preparation of plasmid DNAs

Plasmid DNA from both 1 ml and 200 ml bacterial cultures was prepared by the alkaline lysis method (Birnboim and Doly, 1979). The composition and preparation of all the solutions required for the preparation of plasmid DNAs is described in Table 3.

For screening of colonies, selected clones were grown in 2.5 ml of L-broth containing 100 µg/ml ampicillin in 6 ml vials. After an 18 h incubation at 37°C with vigorous shaking, 1 ml of the cultures was transferred to a 1.5 ml eppendorf tube for plasmid extraction and the remainder was stored at -20°C after the addition of glycerol to 15%. Bacteria were pelleted by centrifugation for 1 min and the supernatant removed using a fine-tip aspirator. The cell pellet was resuspended in 40 µl of solution I (see Table 3). After 5 min on ice, 80 µl of solution II was added and the tube gently vortexed. After a further 5 min on ice, 50 µl of solution III was added and the contents of the

Table 3 Composition and preparation of the solutions required for the preparation of plasmid DNAs

Solution I

50 mM	Glucose
25 mM	Tris-HCl (pH 8.0)
10 mM	EDTA
2 mg/ml	Lysozyme

Prepared when required using crystalline lysozyme and stock solutions of the other components.

Solution II

0.2 M	NaOH
1%	SDS

Prepared when required using stock solutions. To avoid precipitation the solutions were added separately to the appropriate volume of water.

Solution III

3 M	Sodium Acetate (pH 4.6)

Prepared by dissolving 246 g of sodium acetate in the minimum volume of water, titrating to pH 4.6 with glacial acetic acid and then adjusting the volume to 1 litre. 3 M sodium acetate (pH 7.0) was prepared in the same way.

Table 3 contd.

Solution IV

Ribonuclease A at 10 mg/ml

Prepared by gently dissolving 20 mg of crytalline ribonuclease A in 1 ml of distilled wter. After the addition of an equal volume of glycerol the mixture was heated for 10 min in a boiling water bath to destroy any deoxyribonuclease activity and stored in aliquots at -20°C.

The phenol:chloroform mixture was prepared by freshly mixing equal volumes of chloroform and phenol (redistilled phenol equilibrated with 1 mM EDTA, 50 mM β-mercaptoethanol, 0.2 M NaCl, 0.1 M Tris-HCl, pH 8.0).

tube gently mixed by inversion. The tube was incubated on ice for 10 min and then centrifuged for 5 min. This pelleted most of the precipitated protein, high molecular weight RNA and chromosomal DNA and produced an almost clear supernatant containing the plasmid DNA. The supernatant was recovered and transferred to a second centrifuge tube containing 5 µl of solution IV and incubated with shaking at 37°C for 30 min. During the transfer, small amounts of floating material were sometimes carried over, but these were removed at later stages. After two phenol:chloroform (see Table 3) extractions and two chloroform extractions, 500 µl of ethanol was added to the aqueous phase, mixed by vortexing and incubated at -20°C for 16 h to precipitate the plasmid DNA. The precipitate was collected by centrifugation, dissolved in 200 µl of 0.2 M sodium acetate (pH 7) and reprecipitated with 2 volumes of cold ethanol for 8 h at -20°C. The precipitate was again collected by centrifugation and dissolved in 25 µl of distilled water. 5 µl was applied to an agarose gel for electrophoretic analysis and 5 µl aliquots used for restriction analysis.

For the large scale preparation of DNA 2 ml of a 10 ml overnight bacterial culture was used to inoculate 200 ml of L-broth containing ampicillin. This was incubated at 37°C with vigorous shaking untl the culture had reached between 1.0 and 1.5 OD_{600} (typically 3-6 h). Chloroamphenicol was added to a final concentration of 170 µg/ml and incubation continued for a further 20 h. The bacteria were harvested by centrifugation for 15 min at 5,000 rpm in an MSE 6 x 250 ml rotor. The centrifuge pots were drained thoroughly and the bacteria resuspended in 4 ml of solution I before being transferred to a 50 ml Oakridge tube.

This was chilled on ice for 30 min before adding 8 ml of solution II. Mixing was achieved by gently stirring with a glass rod. After chilling on ice for 10 min, 5 ml of solution III was added and the contents of the tube gently mixed by inversion. The tube was incubated on ice for 40 min and the precipitate removed by centrifugation for 15 min at 15,000 rpm in an MSE 8 x 50 ml rotor. The supernatant was collected and transferred to an Oakridge tube containing 50 µl of solution IV and incubated with shaking at 37°C for 30 min. After 2 phenol:chloroform extractions and 2 chloroform extractions the plasmid DNA was ethanol precipitated twice and resuspended in distilled water to a concentration of 1 mg/ml.

All DNA solutions were stored in aliquots at -20°C.

10. Preparation of DNA for injection into oocytes and cultured cells

Since circular DNA is the best template for injection (Gurdon and Melton, 1981) a sucrose gradient was used to purify this form from the plasmid DNA prepared from 200 ml bacterial cultures. The gradient was made by layering 5.5 ml of 10% sucrose (w/v) (in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA) onto an equal volume of 40% sucrose in a 14 ml centrifuge tube. After sealing with 'Nescofilm' the tube was placed horizontally at 37°C for 90 min and then vertically at 4°C for 30 min prior to loading. Approximately 200 µg of DNA was loaded per gradient. The tubes were centrifuged at 34,000 rpm in an MSE 6 x 14 ml rotor for 16 h

at 4°C and fractionated by dropwise collection into 0.5 ml aliquots. The fractions were analysed by agarose gel electrophoresis and those containing the closed circular DNA pooled and diluted with an equal volume of distilled water. After ethanol precipitation the DNA was resuspended in distilled water to a concentration of 1 mg/ml and stored in aliquots at -70°C.

11. Agarose gel electrophoresis of DNA

The solutions required for the agarose gel electrophoresis of DNA are described in Table 4.

Restricted and non-restricted DNA samples were analysed using a mini gel apparatus (Cambridge Biotechnology Laboratories, Cambridge) in the submarine mode. The gel was prepared by adding the required amount of agarose to 38 ml of distilled water in a narrow necked flask. This was weighed, boiled to dissolve the agarose, reweighed and adjusted to the starting weight with distilled water. When the solution had cooled to 50°C 2 ml of 20 x TEA (Table 4) was added and the gel poured. The agarose was dissolved in this way to avoid overheating of the buffer. The electrophoresis buffer was TEA and, prior to loading, the samples were mixed with one-fifth volume of sample buffer. Electrophoresis was at a constant current of 100 mA until the dye front had migrated the required distance. After electrophoresis the gels were stained with ethidium bromide for 30 min and viewed on an ultra-violet light box. Photographic records were made using a Polaroid light camera with

Table 4 Solutions for the agarose gel electrophoresis of DNA

TEA

	Working solution	20x concentrated stock solution (per litre)
Tris	40 mM	96.9 g
Sodium acetate	20 mM	32.8 g
EDTA (disodium salt)	2 mM	14.9 g

A 20x concentrated stock solution was prepared by dissolving the shown amounts of each reagent in the minimum volume of distilled water, titrating to pH 8.3 with glacial acetic acid and adjusting to 1 litre with distilled water.

Sample Buffer

25% sucrose, 0.05% Bromophenol Blue in TEA

Ethidium Bromide

A stock solution at a concentration of 10 mg/ml in water was stored at room temperature in a dark bottle and diluted to 5 µg/ml in TEA when required.

Polaroid type 665 land film.

12. Gel purification of DNA fragments

The gel purification of DNA fragments was done by the method of Girvitz et al. (1980). After electrophoresis, a slit was made in the gel directly in front of the leading edge of the band to be purified. A piece of Whatman 3 MM paper backed with a piece of single dialysis tubing was placed into this slit with the 3 MM paper nearest the DNA band. Electrophoresis was then continued, using the non-submarine mode of operation, until the DNA band had migrated into the paper. The paper and dialysis membrane were removed and placed in a 400 µl microfuge tube which had had a hole made in the bottom using a 26 gauge needle. This tube was placed inside a 1.5 ml tube and centrifuged for 30 sec. The eluate was recovered from the 1.5 ml tube and the paper and dialysis membrane washed several times with TEA. The pooled eluates were extracted twice with phenol:chloroform and twice with chloroform and the DNA was recovered by ethanol precipitation.

13. Preparing oocytes for microinjection

Adult, female Xenopus laevis were obtained from the South African Snake Farm, Windhoek, Cape Province, South Africa and maintained in tanks at 19-21^{o}C. The frogs were fed and cleaned by Mrs. C. Kwasnik.

When a large number of oocytes were required a mature female was killed by the injection of Euthetal (0.3 ml per 100 g of body weight). The complete ovary was then removed via a small incision in the ventral surface of the animal.

When only a few oocytes were required the female was anaesthetised by immersion for about 25 min in a 0.1% solution of MS222, and a small incision was made in the ventral surface of the animal. The required number of ovary lobes were teased out through this hole, ligatured and excised from the animal. The incisions in the skin and body wall were sutured separately and the frog was left to recover partially immersed in 0.5% NaCl for 24 h. This method of obtaining oocytes without killing the frog is not only economical, but has the advantage of allowing the same frog to be used again at a later date, an important consideration since the level of translational product resulting from injected DNA varies between oocytes obtained from different frogs (Asselbergs et al., 1983; Colman, 1984a).

After removal, the ovarian lobes were washed in modified Barths' saline (MBS) (see Table 5) and manually dissected into individual oocytes. These were incubated in modified Barths' saline at 21°C. Only stage V and VI oocytes (Dupont, 1972) were used for injection.

Table 5 Composition and preparation of modified Barths' saline (MBS)

Composition

88.0 mM	NaCl
1.0 mM	KCl
2.4 mM	$NaHCO_3$
15.0 mM	HEPES-NaOH (pH 7.6)
0.30 mM	$CaNO_3 . 4 H_2O$
0.41 mM	$CaCl_2 . 6 H_2O$
0.82 mM	$MgSO_4 . 7 H_2O$
10 µg/ml	Sodium penicillin
10 µg/ml	Streptomycin sulphate

Preparation

To prepare MBS, the solutions were mixed in the following order:

919 ml	of	sterile distilled water
40 ml	of	high salt stock solution A
40 ml	of	divalent cation stock solution B
1 ml	of	antibiotic stock solution C

Solution A High Salt Stock

NaCl	128 g
KCl	2 g
$NaHCO_3$	5 g
HEPES	89 g

Table 5 contd.

These reagents were dissolved in distilled water, titrated to pH 7.6 with 1 M NaOH and adjusted to 1 litre. After autoclaving the solution was stored in 40 ml aliquots at -20°C.

Solution B Divalent Cation Stock

$CaNO_3 \cdot 4\ H_2O$	1.90 g
$CaCl_2 \cdot 6\ H_2O$	2.25 g
$MgSO_4 \cdot 7\ H_2O$	5.00 g

These reagents were dissolved in 1 litre of distilled water, autoclaved and stored in 40 ml aliquots at -20°C.

Solution C Antibiotic Stock

A solution of sodium penicillin and streptomycin sulphate, each at 10 mg/ml was prepared just prior to use.

14. The microinjection and subsequent culturing of oocytes

The equipment and basic manipulations necessary for the microinjection of oocytes is described in Colman (1984b).

a) Injection of radiolabelled cell lysate into oocytes

After centrifugation of the radiolabelled cell lysate to remove any particulate matter, approximately 40 nl of sample was injected into the cytoplasmic region of each oocyte. To minimise the possibility of injecting the sample into the nucleus, and to try to keep the site of injection constant relative to the position of the nucleus, the oocytes were injected as shown in Figure 5. Injected oocytes were incubated in MBS for various times before being enucleated (see below).

b) Injection of mRNA into oocytes

Prior to injection the mRNA was centrifuged at 12,000 g for 2 min at 4°C to remove any particulate matter. Approximately 40 nl of sample was injected into the cytoplasmic region of each oocyte and the injected oocytes were incubated in MBS for 24 h to allow the mRNA to assemble into polysomes. Unhealthy oocytes were discarded and the remainder radiolabelled for 24 h in MBS containing 1 mCi/ml [^{35}S]-methionine in microtitre wells (Flow Laboratories, Irvine, Ayrshire) with 30 µl medium/5 oocytes. The [^{35}S]-methionine was dried under vacuum prior to the addition of the MBS since this was found to increase oocyte viability. After radiolabelling the oocytes were analysed as required.

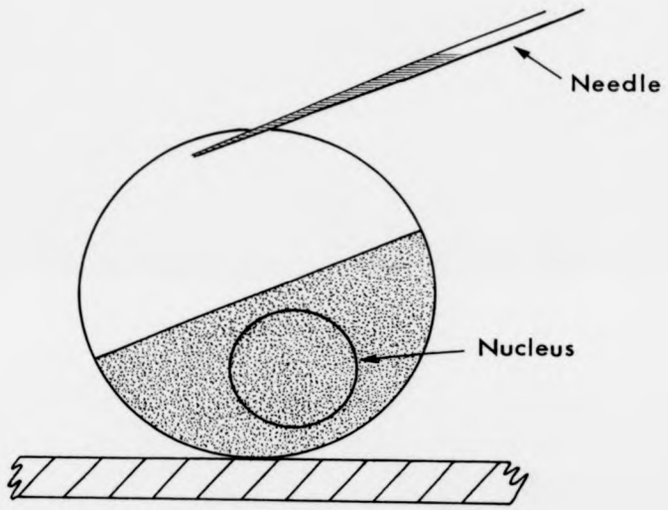


Figure 5 Relative positions of the needle, the nucleus and the oocyte during the injection of radiolabelled cell lysate

c) Injection of DNA into oocytes

Prior to injection the DNA was centrifuged at 12,000 g for 2 min at 4°C to remove any particulate matter. Of the two methods commonly used for injection into the nucleus (Colman, 1984a), the 'blind' injection method was preferred. This involves inserting the needle into the centre of the pigmented region of the oocyte to a depth where the nucleus is believed to be; the relative positions of the needle, the oocyte and the nucleus are shown in Figure 6. This method was preferred to the one which involves centrifugation to push the nucleus to the oocyte surface since it was more difficult to isolate intact nuclei from the centrifuged oocytes. We were also concerned that the change in both the shape of the nucleus and its position in the cell caused by the centrifugation might affect its ability to transport and accumulate proteins.

The success of the 'blind' injection method was estimated by the use of a solution of Trypan Blue in PBS (see Colman, 1984a), which showed that in regular practice sessions over 90% of the nuclei were being successfully injected.

The survival of the oocytes was increased when they were kept at 4°C for 30 min both before and after injection. Injected oocytes were incubated in MBS for 24 h to allow the assembly of chromatin and the accumulation of mRNA before being radiolabelled for 6 h in MBS containing [^{35}S]-methionine at a concentration of 1 mCi/ml. After radiolabelling the oocytes were analysed as required.

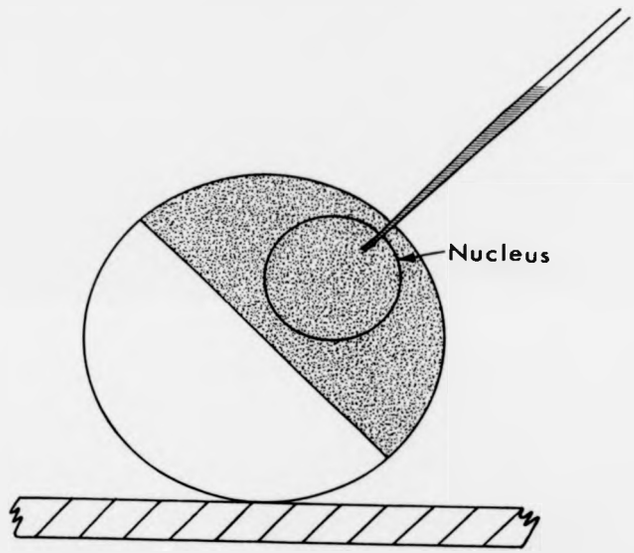


Figure 6 Relative positions of the needle, the oocyte and the nucleus when injecting DNA by the 'blind' method

In some experiments it was necessary to perform an unlabelled chase on the radiolabelled oocytes. This was achieved by thoroughly washing the oocytes in MBS containing 10 mM methionine and then incubating in the same medium for the desired length of time before analysis.

Each oocyte was injected with 25 nl of DNA at a concentration of 150 µg/ml since this was found to give the maximum expression of protein (see 'Results', Section II).

d) Preparation of radiolabelled cloned NP protein for injection into oocytes

Sixty oocytes injected with the plasmid pTKNP were radiolabelled and enucleated as described elsewhere in the methods. The enucleated oocytes were drained of excess medium, disrupted with a glass rod and centrifuged at 12,000 g for 3 min at 4°C. This gave a soluble cytoplasmic fraction containing radiolabelled cloned NP (cyt-NP). The nuclei were collected in the minimum volume of MBS and disrupted by passage through a 50 µl Hamilton syringe to produce a nuclear fraction containing radiolabelled cloned NP (nuc-NP). These samples were not frozen but kept on ice before being injected into freshly isolated oocytes (see below).

e) Injection of radiolabelled cloned NP protein into oocytes

Both the cyt-NP and nuc-NP samples were centrifuged at 12,000 g for 2 min at 4°C prior to injection into oocytes. The samples were injected within 2 h of preparation. Injection into the cytoplasmic or nuclear regions of oocytes was as described above except that 25 nl of sample

was used in each case. The injected oocytes were incubated in MBS for the required time and then analysed.

15. Enucleation of oocytes

Oocytes were enucleated in MBS at 4°C by holding the oocyte with a pair of watchmaker's forceps and making a slit in the apex of the pigmented animal half with the tip of a 26-gauge syringe needle. During this puncturing it was important that the needle did not penetrate into the cytoplasm since this would be likely to damage the nucleus. The oocyte was then gently squeezed around its middle to extrude the nucleus (Figure 7). Any cytoplasmic material adhering to the nucleus was removed by pipetting it up and down in a wide-mouthed plastic micropipettor tip. Isolated nuclei were collected in a minimum volume of saline and transferred to an eppendorf tube on ice. The enucleated oocytes were transferred using watchmakers forceps to a separate tube which was also kept on ice. Both samples were homogenised immediately after isolation.

From start to finish the enucleation procedure took about 20 sec and the loss of proteins during isolation was minimal as estimated from the analysis of the protein content of total oocytes and the combined cytoplasmic and nuclear fractions.

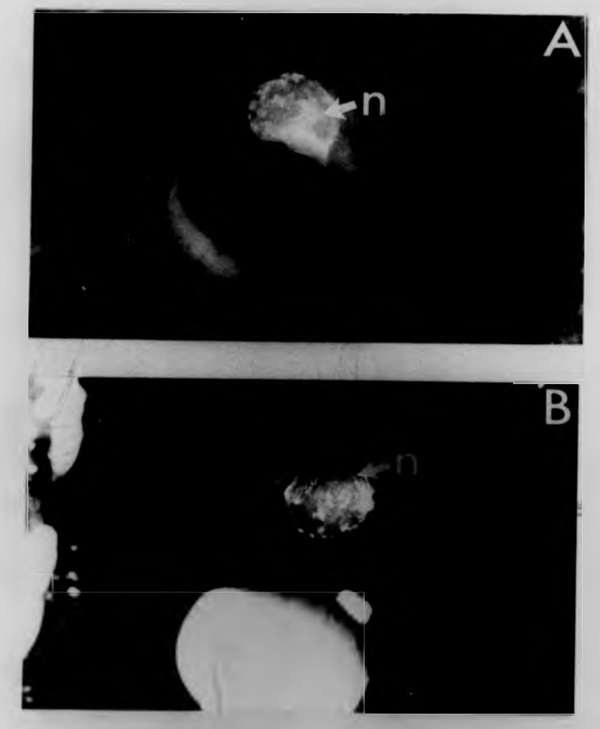


Figure 7 Stages in the enucleation of an oocyte

A superficial slit was made in the apex of the pigmented half of the oocyte. By gently squeezing the oocyte around the equitorial region the nucleus was gradually extruded (A) and eventually became free.

Figure 7 Stages in the enucleation of an oocyte

A superficial slit was made in the apex of the pigmented half of the oocyte. By gently squeezing the oocyte around the equitorial region the nucleus was gradually extruded (A) and eventually became free.

16. Homogenisation of oocytes and oocyte fractions

Total oocytes and enucleated oocytes were homogenised at 4°C in homogenisation buffer (HB) (see Table 6) at 60 µl per fraction and clarified by centrifugation at 12,000 g for 1 min at 4°C. This gave three layers which, from the bottom, were a pellet of yolk platelets and pigment granules, a soluble fraction and a lipid pellicle. The middle soluble fraction was collected using a drawn out glass pipette and stored at -20°C.

Nuclei were disrupted with ultrasound from a probe at 1.5 A at 4°C in 60 µl HB per nucleus. This was stored at -20°C and, when required, the thawed sample was resonicated prior to analysis.

17. Immunoprecipitation of oocyte fractions

440 µl of immunoprecipitation buffer A (Table 6) was added to 60 µl aliquots of oocyte sample. After addition of antibody the mixture was incubated at 26°C for 1 h. During this time a sample of the frozen S. aureus (see Section 18 of 'Methods A') was thawed at 37°C, pelleted by centrifugation at 12,000 g for 30 sec and resuspended to 10% (v/v) in immunoprecipitation buffer B (Table 6). Resuspension was attained by repeatedly sucking up and down using an eppendorf micropipette. This suspension was left at room temperature for 15 min, repelleted and washed three more times in buffer B. The washed pellet was resuspended to 10% (v/v) in buffer B and 100 µl was added to the antibody/sample

Table 6 Composition and preparation of solutions for oocyte homogenisation, immunoprecipitation and polyacrylamide gel electrophoresis (PAGE)

Homogenisation buffer

10 mM	Tris-HCl (pH 7.4)
1 mM	PMSF*
1%	NP40

*PMSF stock solution: 0.1 M in ethanol. Stored at -20°C and dispensed just prior to use.

Cracking buffer

25%	Glycerol
10%	SDS
0.01%	Bromphenol blue
3.5 M	β-mercaptoethanol

Immunoprecipitation buffer A

0.1 M	Tris-HCl (pH 8.2)
0.1 M	KCl
5 mM	$MgCl_2$
1%	Triton X-100
1%	Sodium deoxycholate
0.5%	SDS
1mM	PMSF

Table 6 contd.

Immunoprecipitation buffer B

0.1 M	Tris-HCl (pH 8.2)
0.1 M	KCl
5mM	$MgCl_2$
1%	Triton X-100
1mM	PMSF

The immunoprecipitation buffers were prepared by mixing stock reagents in the following order:

	Buffer A	Buffer B
1 M Tris-HCl (pH 8.0)	10 ml	10 ml
1 M KCl	10 ml	10 ml
1 M $MgCl_2$	0.5 ml	0.5 ml
10% Triton X-100	10 ml	10 ml
10% Sodium deoxycholate	10 ml	-
10% SDS	5 ml	-
0.1M PMSF	1 ml	1 ml

Each was adjusted to 100 ml with distilled water. The pH of each solution was now 8.2

mixture. The mixture was incubated on a slowly rotating wheel at 4°C for 16 h and the bacteria were then pelleted and washed five times with fresh immunoprecipitation buffer A. The washed pellets were resuspended by vortexing in a mixture of 4 parts 10 mM Tris (pH 7.4) to 1 part cracking buffer. The sample was boiled for 2 min, vortexed, boiled for a further 2 min and centrifuged at 12,000 g for 2 min prior to analysis by SDS-PAGE.

Sufficient antibody was added to the immunoprecipitates to precipitate all of a protein in a particular sample.

18. Growth and treatment of Staphylococcus aureus for use in immunoprecipitation

This followed the method of Kessler (1975). A 10 ml overnight culture of Staphylococcus aureus Cowan 1 strain (kindly provided by H. Killen, University of Warwick) was used to inoculate 1 litre of growth medium (see Table 7) in a 4 litre standard Erlenmeyer flask. This was incubated with shaking at 37°C for 20 h. The bacteria were pelleted by centrifugation at 8,000 g for 10 min and washed twice in PBS containing 0.05% (w/v) sodium azide. The washed pellet was resuspended to 10% (w/v) in PBS-azide containing 1.5% formalin and stirred for 90 min at 23°C. The bacteria were pelleted, washed twice in PBS-azide and resuspended to 10% (w/v) in the same solution. They were then added to a large Erlenmeyer flask to a depth of less than 1.5 cm and killed by rapid swirling for 5 min in an 80°C water bath followed by rapid cooling

Table 7 Composition and preparation of the growth medium for Staphylococcus aureus

Penassay - broth	17.5 g
Difco - yeast extract	2.5 g
Casamino acids	5.0 g

Made to 1 litre with distilled water and titrated to pH 7.2.

(The addition of β-glycerophosphate, niacin and thiamine-HCl (Kessler, 1975) did not increase the cell yield or their ability to bind antibodies and so were omitted from the growth medium).

in an iced water bath. After 2 more washes in PBS-azide the concentration was set at 10% (v/v) with the aid of a micrometer. The bacteria were stored in 1 ml aliquots at -70°C.

19. Analysis of unimmunoprecipitated oocyte fractions

One volume of cracking buffer (see Table 6) was added for every 4 volumes of sample. The sample was vortexed, boiled for 3 min and analysed by SDS-PAGE (see below).

20. Polyacrylamide gel electrophoresis (PAGE) of proteins

In most cases electrophoresis used linear gradient polyacrylamide gels containing 0.1% sodium dodecyl sulphate (SDS) (Cook et al., 1979a) and the buffer system of Laemmli (1970). The polyacrylamide gradient was from 10-30% (w/v) and was stabilized by 0 to 8% glycerol. The reagents, and their relevant amounts, used in the preparation of these gels are shown in Table 8. Gradients were prepared using a twin chambered gradient maker and poured under gravity. To obtain a level surface to the gel it was overlaid with water-saturated butan-1-ol during polymerisation. The ammonium persulphate concentrations were designed such that acrylamide polymerisation started at the lowest concentration of acrylamide (i.e. the top of the gel) and proceeded downwards. This was to minimise disturbance to the gradient through heating effects. Once polymerised (typically 90 min at room temperature) the butan-1-ol

Table 8 Solutions for the preparation of gradient polyacrylamide gels

Reagents	Volume (ml)	Molar or % concentration	Volume (ml)	Molar or % concentration
60% Acrylamide plus	4.16	10% Acrylamide	12.50	30% Acrylamide
0.282% Bis-acrylamide		0.047% Bis-acrylamide		0.141% Bis-acrylamide
1 M Tris-HCl (pH 8.6)	9.30	0.37 M	9.30	0.37M
Water	10.93		0.25	
10% SDS	0.25	0.1%	0.25	0.1%
80% Glycerol	-	-	2.50	8%
1% Ammonium persulphate	0.33	0.0132%	0.165	0.0066%
TEMED	0.033		0.033	

was removed and the top of the gel washed extensively with distilled water. The stacking gel (see Table 9) was added and allowed to polymerise for 20 min before use. Electrophoresis (see Table 9 for electrophoresis buffer) was at 14 mA for 16 h.

In some cases single concentration gels were used. The reagents, and their relevant amounts, used in the preparation of these gels are shown in Table 10. The electrophoresis buffer used for these gels was the same as described in Table 9. Electrophoresis was at 14 mA until the dye front had reached the bottom of the gel.

Gels with sufficient radioactivity were dried under suction onto filter paper and exposed to Fuji RX X-ray film. If increased sensitivity was required the gels were fluorographed (Bonner and Laskey, 1974) and exposed to preflashed X-omat S film at -70°C.

21. Determination of the amount of radioactivity in protein bands

After exposing for autoradiography, the gels were aligned with the developed film and the positions of the relevant bands marked. These were excised from the gel and placed into 3 ml of gel slice scintillant. This comprised, per litre: 92 ml of NCS tissue solubiliser, 42 ml of liquifier (4 g PPO and 50 mg POPOP in toluene), 856 ml of toluene and 10 ml of water. Slices were incubated for 48 h at 37°C and 6 h at 4°C before measuring the radioactivity.

Table 9 Solutions used in gradient polyacrylamide gel electrophoresis

Stacking gel

Reagent	Volume (ml)	Molar or % concentration
30% Acrylamide plus 0.8% Bis-acrylamide	1.50	4.5% Acrylamide 0.12% Bis-acrylamide
1 M Tris-HCl (pH 6.8)	1.25	0.125M
Water	7.09	
10% SDS	0.10	0.1%
10% Ammonium persulphate	0.03	0.03%
TEMED	0.03	

Electrophoresis Buffer

Reagent	Molar or % concentration	
Tris	0.025M	14.8 g
Glycine	0.197M	3.0 g
SDS	0.1%	1.0 g

Prepared by dissolving the reagents in 1 litre of distilled water.

Table 10 Solutions used in single concentration polyacrylamide gel electrophoresis

Reagent	Concentration of acrylamide			
	7%		15%	
	Volume (ml)	Molar or % concentration	Volume (ml)	Molar or % concentration
30% Acrylamide plus 0.8% Bis-acrylamide	11.66	7% Acrylamide 0.19% Bis-acrylamide	25.00	15% Acrylamide 0.5% Bis-acrylamide
3M Tris-HCl (pH 8.8)	6.00	0.36M	6.00	0.36M
Water	31.62		18.28	
10% SDS	0.50	0.1%	0.50	0.1%
10% Ammonium persulphate	0.20	0.04%	0.20	0.04%
TEMED	0.02		0.02	

Table 10 (cont'd.)

Stacking gel

Reagent	Volume (ml)	Molar of % concentration
30% Acrylamide plus 0.8% Bis-acrylamide	4.00	6% Acrylamide 0.16% Bis-acrylamide
1M Tris-HCl (pH 6.8)	2.50	0.125M
Water	13.18	
10% SDS	0.20	0.1%
10% Ammonium persulphate	0.10	0.05%
TEMED	0.015	

22. Calculation of the nuclear to cytoplasmic concentration ratio

Calculation of the extent to which a protein is concentrated in the nucleus depends on the relative nuclear and cytoplasmic volumes. On a purely volumetric basis the nucleus occupies about 4% of fully grown amphibian oocytes, but if such a value is used almost any substance that is able to cross the nuclear envelope appears to accumulate slightly in nuclei *in vivo*, i.e. over 4% of the substance is found in the nucleus. These substances include inorganic ions, sugars, amino acids, gold particles and small proteins (see Bonner, 1978 for references). A possible reason for this surprising observation was first proposed by Abelson and Duryee who suggested that the low level accumulation of exchangeable sodium ions in oocyte nuclei (where approximately 8% was in the nucleus) was the result of different water contents in the nucleus and the cytoplasm (Abelson and Duryee, 1949). It was subsequently demonstrated that the relative water content between the nucleus and the cytoplasm in these cells was approximately 1.9:1 (Century *et al.*, 1970). This would explain the nuclear presence of about 7.6% (1.9 x 4) of the sodium ions.

Even allowing for these different water contents many substances were still found to accumulate at low levels in the nucleus. When tritiated sucrose, for example, was injected into amphibian oocytes approximately 12% was found in the nucleus (Horowitz, 1972). This gives a nuclear to cytoplasmic concentration ratio of 3 when only the relative nuclear and cytoplasmic volumes are considered, and a ratio of 1.6 when the relative water contents are also taken into account. An earlier suggestion that

this may reflect the inability of some of the cytoplasmic water to act as a solvent (Horowitz and Fenichel, 1968) was verified by an elegant experiment in which a defined aqueous reference phase was introduced into the cytoplasm (Horowitz and Paine, 1976). When sucrose was injected into these oocytes it accumulated in both the nucleus and the reference phase to a similar extent, and using this result these workers concluded that about 30% of the cytoplasmic water was not available to act as a solvent. This phenomenon of cytoplasmic exclusion is not restricted to oocytes and is believed to be responsible for low level nuclear accumulation in amoebae (Feldherr, 1962b) and the giant salivary gland cells of Chironomous (Paine, 1975).

Although these observations show how cytoplasmic exclusion can account for low level nuclear accumulation they do not specify the molecular mechanism(s) responsible. These mechanisms may include changes in the bulk solvent properties of the water, for example, through hydrophobic interactions or solvent-polymer hydrogen bonding, or they could involve restricted accessibility of the water by micro-compartmentalisation or polymer-solute repulsion. Similar behaviour is seen in other macromolecular systems such as solutions of vaccinia virus (McFarlane, 1939), hyaluronic acid (Ogston and Phelps, 1960) and the cross-linked dextrans used in chromatography.

In amphibian oocytes another factor needs to be considered. The cytoplasm of these cells contains a large number of yolk platelets which contribute to the excluded volume as they are impermeable to injected proteins (Bonner, 1975a).

Taking all these factors into consideration it has been estimated that the nucleus represents 12% of the oocyte volume accessible to most macromolecules (Bonner, 1978).

23. Injection of the plasmid pTKNP into cultured cells

The injection of the plasmid pTKNP into cultured cells was performed by Dr. A. Colman using the method of Graessmann et al. (1980). BHK-21 cells were grown on ethanol washed, heat-sterilised 16 mm glass coverslips in 5 cm plastic Petri dishes. The plates were seeded at 1-2 x 10^6 cells per plate and allowed to grow to 30-50% confluency prior to injection with DNA at a concentration of 1 mg/ml.

24. Fluorescent antibody staining of cultured cells

Cells injected with the plasmid pTKP or infected with influenza virus A/NT/60/68 were incubated for 8 h prior to analysis by indirect fluorescent antibody staining. This analysis was performed by Miss E. Wallis using the method of Ash et al. (1977). Fluorescence was observed with a Zeiss microscope (Micro-Instruments Ltd., Oxford).

METHODS B

THE CONSTRUCTION OF PLASMIDS

1. Construction of the plasmid pTKNP

The construction and basic features of the eukaryotic expression vector pTK_2 is described in Krieg et al. (1984) and Figure 8.

Influenza virus contains a genome consisting of eight segments of single stranded RNA which is of opposite polarity to mRNA and therefore termed negative sense (Baltimore, 1971). The majority of these segments encode a single polypeptide (see 'General Introduction', Section VII) and the fifth largest segment, band 5, encodes the nucleoprotein (NP). A full length double stranded DNA copy of band 5 of influenza virus A/NT/60/68 was kindly provided by Professor G. G. Brownlee (Huddleston and Brownlee, 1982) and inserted into the Pvu II site of the plasmid pAT/Pvu II/8 (Anson et al., 1984) to produce pATNP. The NP coding region of pATNP was excised and inserted into the vector pTK_2 to give pTKNP (Figure 9).

To ease description of the manipulations of pTKNP a numbering system was employed. The reference origin is taken as being midway through the Pvu II site at the junction between the pBR322 and TK promotor fragments. Numbering is in the direction of transcription from the TK promotor and gives a total of 5423 bp.

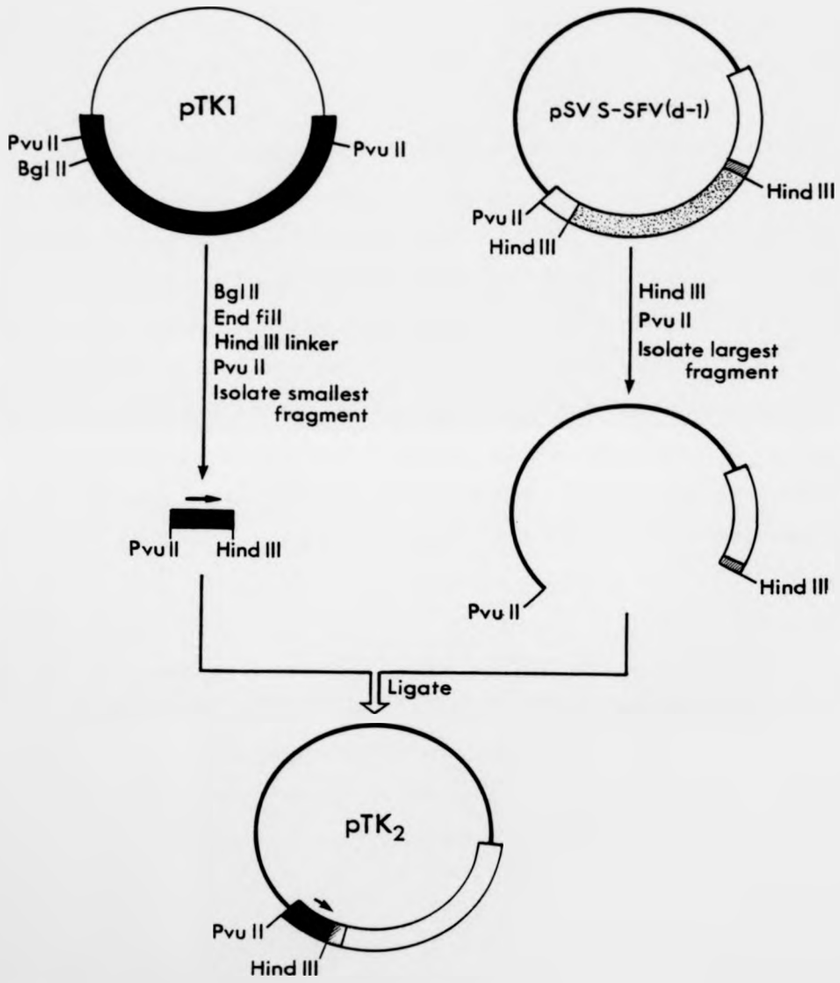


Figure 8 Construction of the expression vector pTK_2

The plasmid pSV S-SFV (d-1) (Kondor-Koch et al., 1983) contains part of the Semliki Forest virus (SFV) genome (stipled box) under the expressional control of elements from the simian virus (SV) 40 genome (open box). The SV40 early promotor (betwen the Pvu II and Hind III sites) and the SFV sequence were removed by digestion with Pvu II and Hind III and replaced by the Pvu II/Bgl II fragment of pTK1. The plasmid pTK1 consists of a fragment of the herpes simplex virus (HSV) genome (solid box) containing the thymidine kinase (TK) gene cloned into the Bam HI site of pAT 153 (thin line) (Wilkie et al., 1980). The resulting vector, pTK_2, contains the 2.3 Kbp fragment of plasmid pBR322 from the Eco RI site at position 4360 to the Pvu II site at position 2067 (Sutcliffe, 1978) (thick line). This carries the origin for DNA replication and the gene conferring resistance to ampicillin; the 988 bp Eco RI/Bcl I fragment of the SV40 genome (open box) containing the polyadenylation signal for the SV40 early transcripts (Tooze, 1980); a 250 bp Pvu II/Bgl II fragment of the plasmid pTK1 (shaded box), containing the transcription promotor region of the TK gene but lacking the ATG translation initiation codon. The direction of transcription is indicated by the arrow. A 21 bp synthetic oligonucleotide containing translation termination codons in all three reading frames (Pettersson et al., 1983) (hatched box). In future this region is termed the stop region.

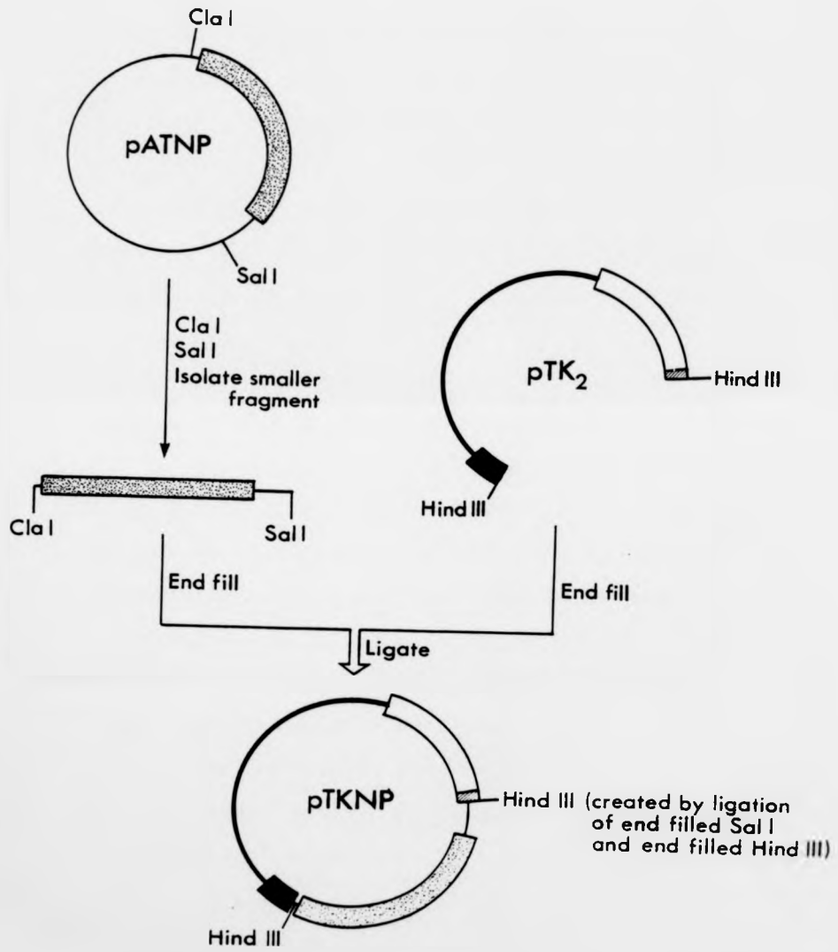


Figure 9 Construction of the plasmid pTKNP

A full length double stranded DNA copy of the nucleoprotein gene of influenza virus A/NT/60/68 (stipled box) was blunt end ligated into the Pvu II site of pAT/Pvu II/8 to produce pATNP. The NP coding region was excised from pATNP using Cla I and Sal I and the fragment was end-filled using the Klenow fragment of Escherichia coli DNA polymerase prior to blunt end ligation into the end-filled Hind III site of the expression vector pTK_2 to give pTKNP. The elements in pTKNP have the same key as those for pTK_2 in Figure 8 with the addition of the NP gene (stipled box) and the flanking sequences from pAT/Pvu II/8 (thin line).

2. Construction of mutants plasmids lacking various amounts of the carboxyl terminus of the NP gene

In the production of these mutants use was made of the Hind III restriction site created when the end-filled Sal I site of the NP gene containing insert was ligated to the end-filled Hind III site of the vector pTK_2 (see Figure 9). This Hind III site is therefore immediately before the stop region of pTKNP. The idea was to remove from pTKNP a region from a chosen restriction site within the NP coding sequence to this Hind III site and thereby remove that region of NP to the carboxyl side of the chosen site. Since such a manipulation removes the authentic NP translation terminator the resulting mutants are terminated by codons in the stop region. Six clones were constructed using this procedure:

a) Construction of the plasmid pTKNP Hind 1348

The plasmid pTKNP contains 3 Hind III sites; one in the short pAT/Pvu II/8 sequence at the amino end of the NP gene (at position 263); one within the NP coding region (1348) and a third which is recreated at the carboxyl end of the NP insert (2118). A Hind III partial digestion of pTKNP was performed and the 4653 bp fragment from Hind III (2118) to Hind III (1348) was purified by gel electrophoresis. This was ligated to give pTKNP Hind 1348. These manipulations are illustrated diagrammatically in Figure 10 while Figure 11 shows the relevant manipulations at the nucleotide level.

The predicted protein from pTKNP Hind 1348 has the first 345 amino acids of NP plus 4 amino acids encoded by the stop region. From the nucleotide sequence it has a MW of 39,405.

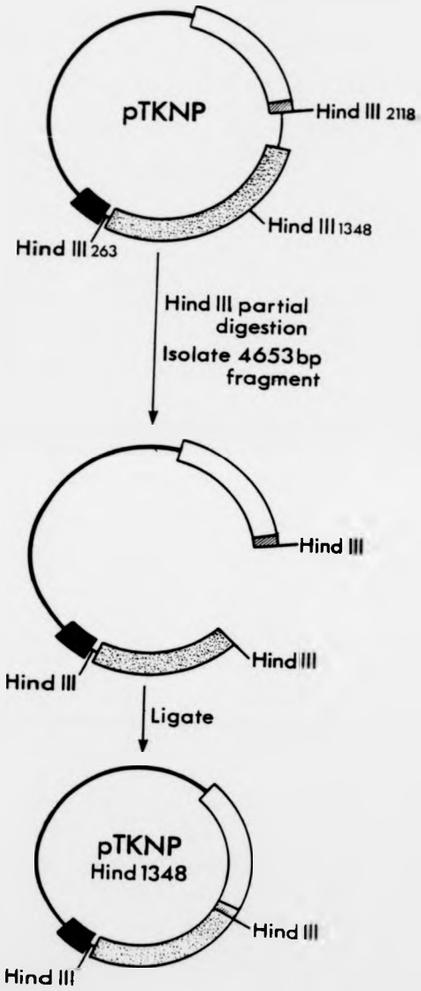


Figure 10 Construction of the plasmid pTKNP Hind 1348

See text for details of the construction. The key for the various elements is:

Thick line	pBR322 fragment
Solid box	TK promotor
Thin line	Flanking sequence from pAT/Pvu II/8
Stipled box	NP coding sequence
Hatched box	Stop region
Open box	SV40 polyadenylation signal

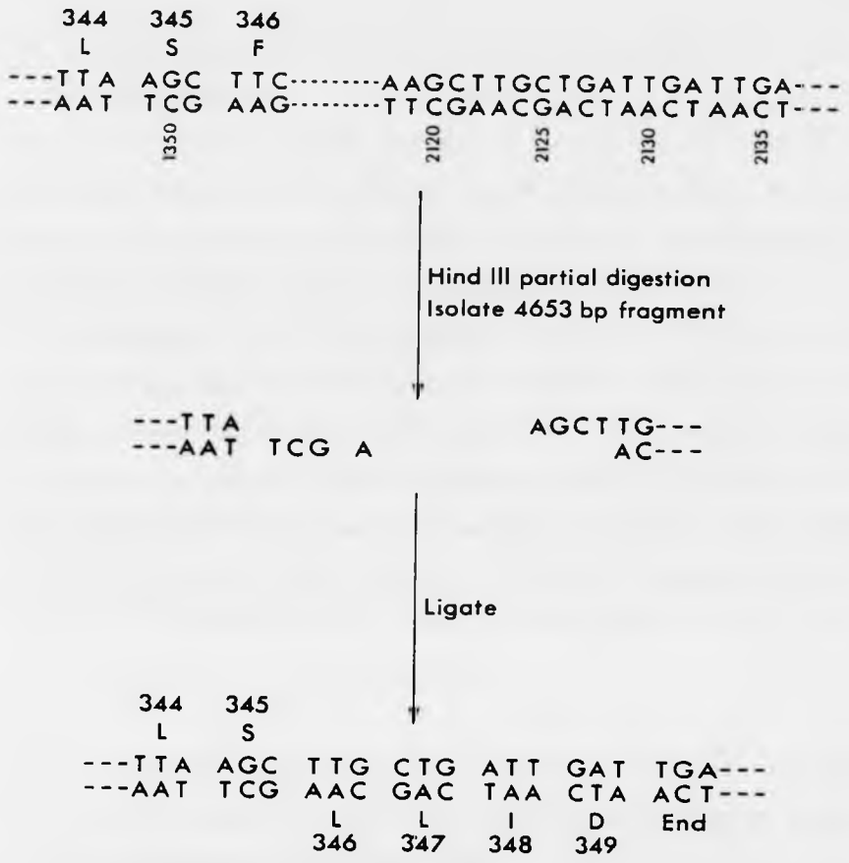


Figure 11 Construction of the plasmid pTKNP Hind 1348 at the nucleotide level

Only those regions around the Hind III sites at positions 1348 and 2118 are shown. The numbers below the top sequence refer to the positions of the nucleotides in the plasmid pTKNP while above the sequence are the encoded amino acids and their positions in NP. The letters and numbers below the bottom sequence refer to the amino acids encoded by the stop region and their positions in the mutant protein.

b) Construction of the plasmids pTKNP Pvu 705, pTKNP Pvu 1011 and pTKNP Pvu 1295

The plasmid pTKNP contains four Pvu II sites; the one used as the reference origin and three within the NP coding region. The relative positions of these sites are shown in Figure 12. After partial digestion with Pvu II three DNA fragments were purified by electrophoresis: the 708 bp fragment from Pvu II (-3) to Pvu II (705); the 1014 bp fragment from Pvu II (-3) to Pvu II (1011) and the 1298 bp fragment from Pvu II (-3) to Pvu II (1295). To accomodate these fragments the plasmid pTKNP was digested with Pvu II and Hind III and the resulting 3305 bp fragment from Hind III (2118) to Pvu II (-3) purified and end filled. Ligation of the three fragments to this manipulated vector resulted in the plasmids pTKNP Pvu 705, pTKNP Pvu 1011 and pTKNP Pvu 1295 respectively.

Figure 13 shows the construction of pTKNP Pvu 705 at the nucleotide level while figures 14 and 15 show the final constructs of pTKNP Pvu 1011 and pTKNP Pvu 1295 respectively.

The predicted protein from pTKNP Pvu 705 has 134 amino acids, the first 130 of NP and 4 encoded by the stop region, and a MW of 15,450. The predicted protein from pTKNP Pvu 1011 has 236 amino acids, the first 232 amino acids of NP and 4 encoded by the stop rgion, and a MW of 26,983. The predicted protein from pTKNP Pvu 1295 has 332 amino acids, the first 327 amino acids of NP and 5 encoded by the stop region, and a MW of 37,487.

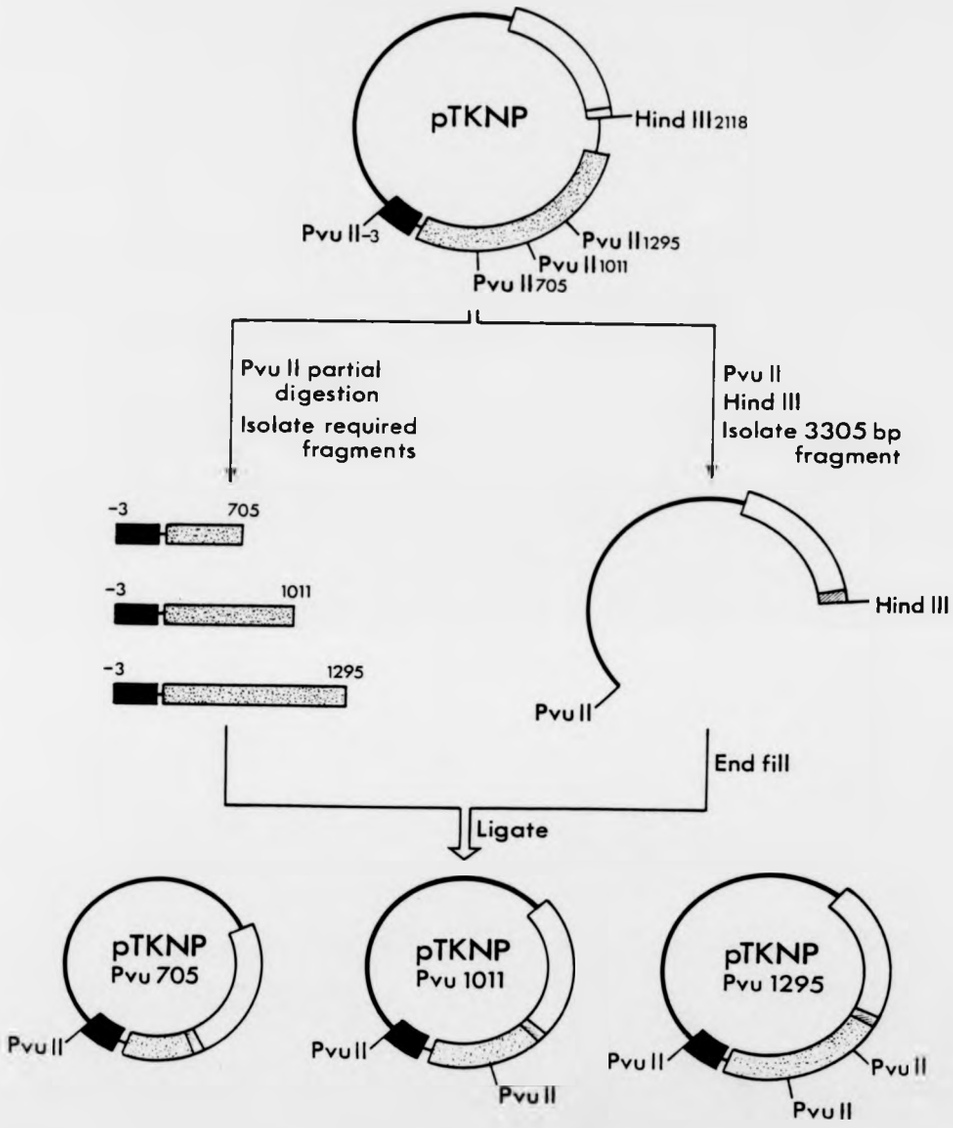


Figure 12 Construction of the plasmids pTKNP Pvu 705, pTKNP Pvu 1011 and pTKNP Pvu 1295

See text for details of the construction. The key for the various elements is as in Figure 10.

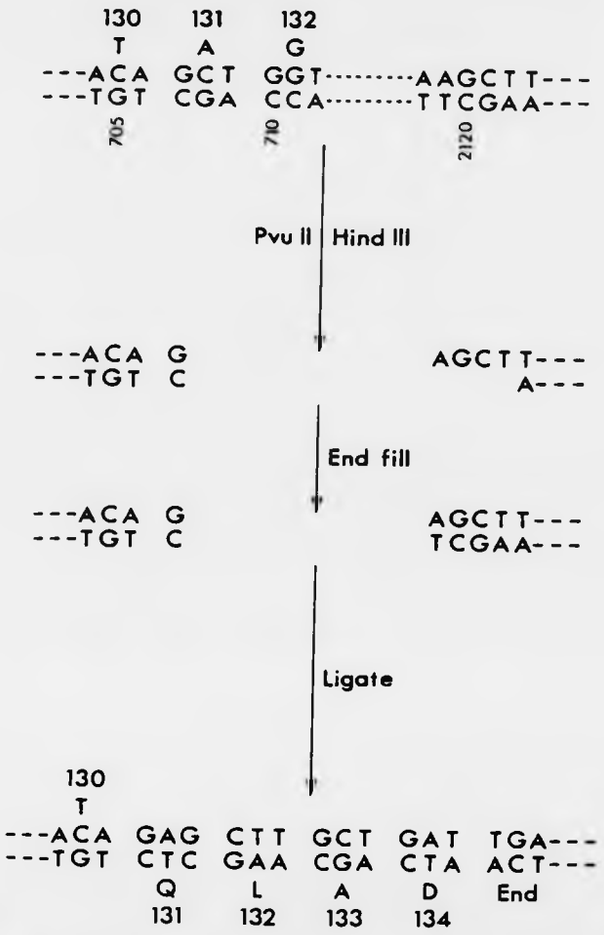


Figure 13 Construction of the plasmid pTKNP Pvu 705 at the nucleotide level

Only those regions around the Pvu II site (705) and the Hind III site (2118) are shown. The numbers below the top sequence refer to the positions of the nucleotides in the plasmid pTKNP while above the sequence are the encoded amino acids and their positions in NP. The letters and numbers below the bottom sequence refer to the amino acids encoded by the stop region and their positions in the mutant protein.

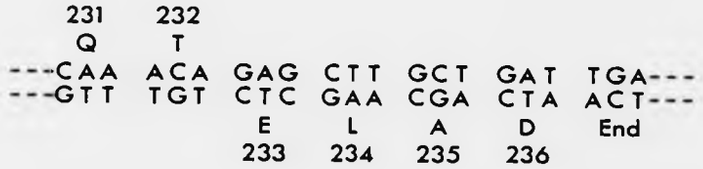


Figure 14 The carboxyl terminus of the NP encoding region of the plasmid pTKNP Pvu 1011

This shows the result of ligating the Pvu II site at position 1011 to the end- filled Hind III site (2118). Above the sequence are the encoded amino acids and their positions in NP while below the sequence are the amino acids encoded by the stop region and their positions in the mutant protein.

```
      326   327
       S     Q
---AGT  CAG  AGC  TTG  CTG  ATT  GAT  TGA---
---TCA  GTC  TCG  AAC  GAC  TAA  CTA  ACT---
             S    L    L    I    D   End
            328  329  330  331  332
```

Figure 15 The carboxyl terminus of the NP encoding region of the plasmid pTKNP Pvu 1295

This shows the result of ligating the Pvu II site at position 1295 to the end-filled Hind III site (2118). Above the sequence are the encoded amino acids and their positions in NP while below the sequence are the amino acids encoded by the stop region and their positions in the mutant protein.

c) Construction of the plasmid pTKNP Acc 1250

Due to the presence of inconvenient restriction sites in pTKNP, the plasmid pATNP was used as the starting point in the construction of pTKNP Acc 1250. As shown in Figure 16 pATNP has two Acc I and one Cla I restriction sites. Digestion of pATNP with Acc I and Cla I resulted in the production of a 993 bp fragment which was purified, end filled and blunt end ligated into the end filled Hind III site of the vector pTK_2 (Figure 16).

These manipulations are shown at the nucleotide level in Figure 17. The resulting clone is identical to pTKNP at the amino end of the insert (see Figure 9). The predicted protein has the first 312 amino acids of NP and a MW of 35,212.

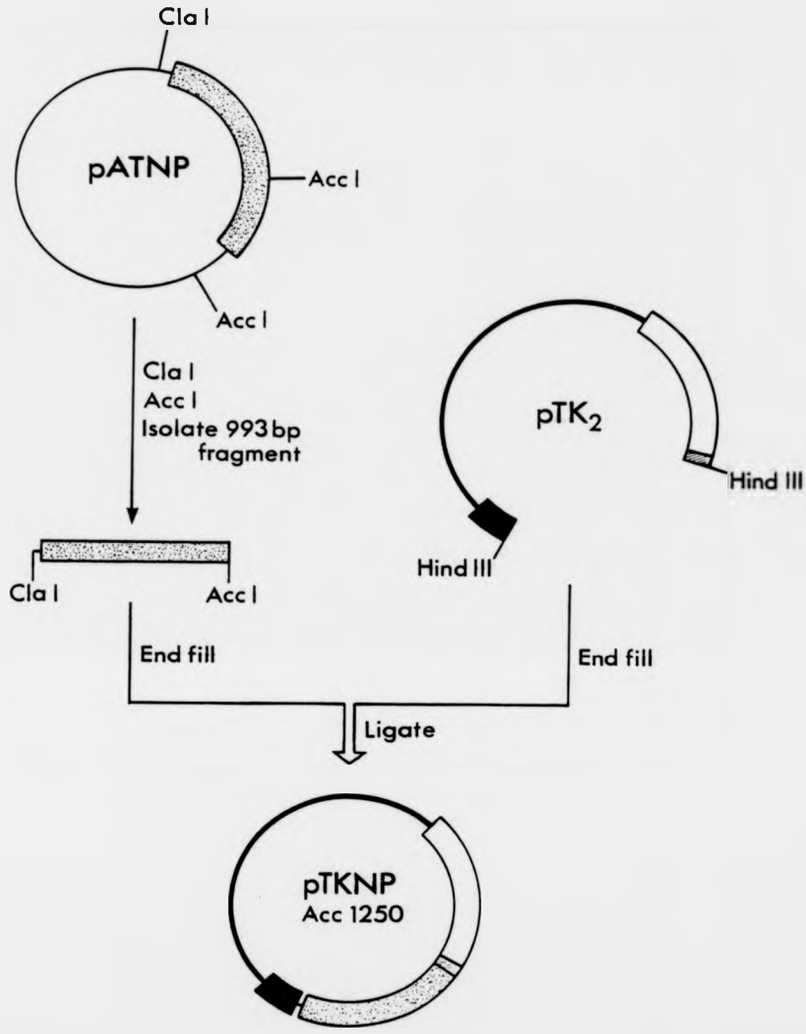


Figure 16 Construction of the plasmid pTKNP Acc 1250

See text for details of the construction. The key for the various elements is as in Figure 10.

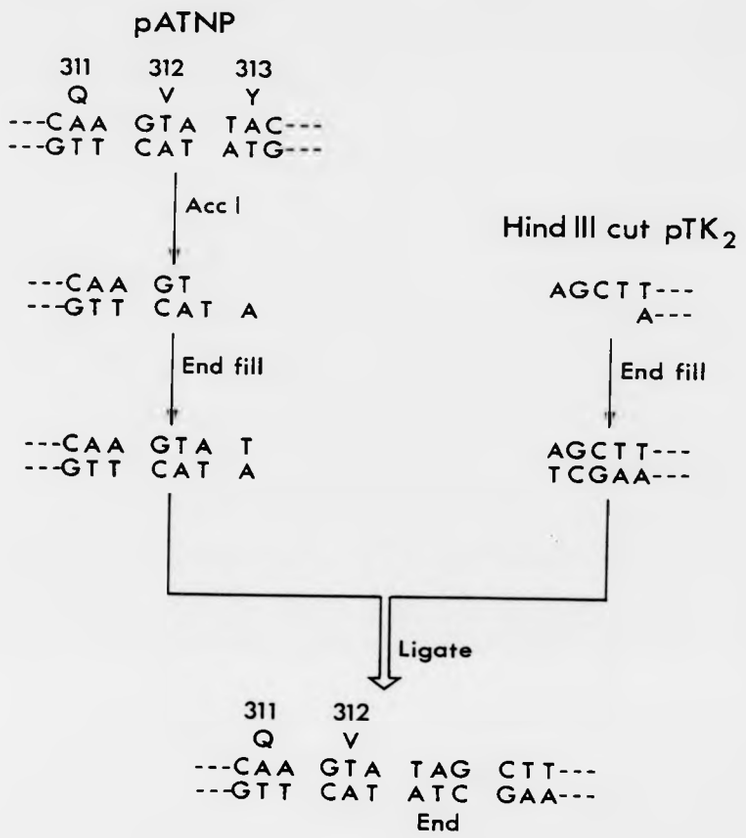


Figure 17 Construction of the plasmid pTKNP Acc 1250 at the nucleotide level

The encoded amino acids and their positions in NP are shown at the relevant Acc I site in the plasmid pATNP. In the final construct the translation termination codon from the stop region is also shown.

d) Construction of the plasmid pTKNP Hae 1474

Due to the presence of inconvenient restriction sites in pTKNP the plasmid pATNP was used as the starting point in the construction of pTKNP Hae 1474. Figure 18 shows the position of the unique Cla I site in pATNP and the first Hae III site in a clockwise direction from this Cla I site. The other twentyone Hae III sites are omitted for clarity. Digestion of pATNP with Hae III and Cla I resulted in the production of a 1217 bp fragment which was purified, end filled and blunt end ligated into the end filled Hind III site of the vector pTK_2 (Figure 18). These manipulations are shown at the nucleotide level in Figure 19. The resulting clone is identical to pTKNP at the amino end of the insert (see Figure 9). The predicted protein has 390 amino acids, the first 386 of NP and 4 encoded by the stop region, and a MW of 44,029.

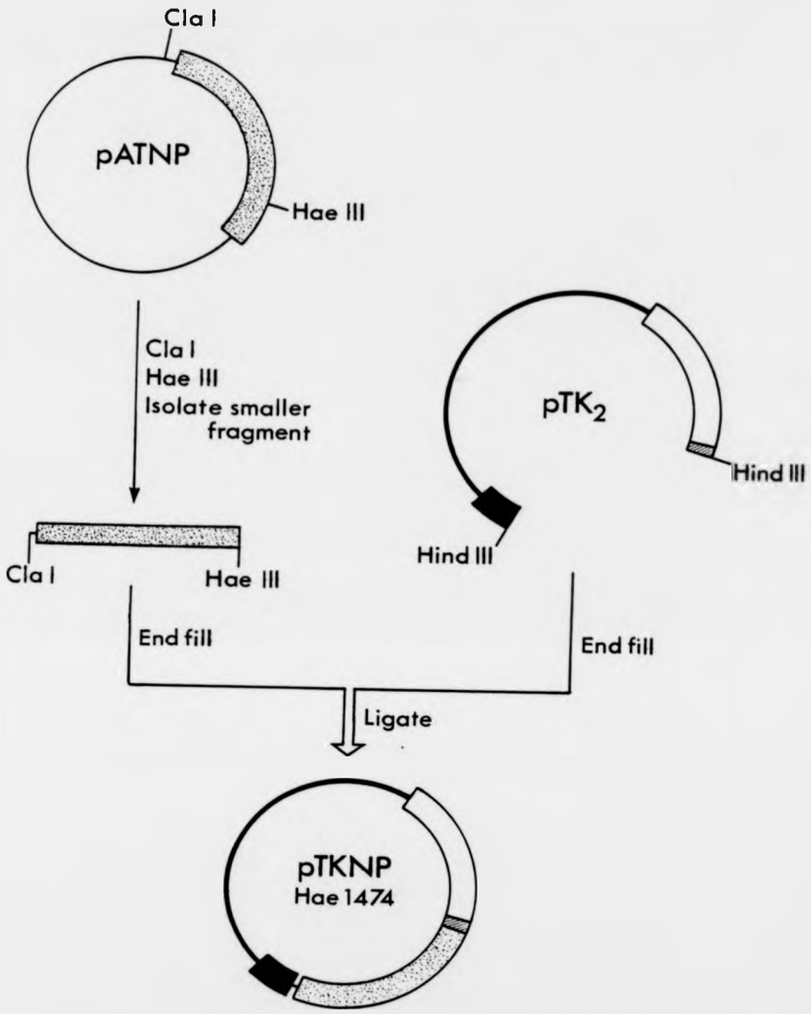


Figure 18 Construction of the plasmid pTKNP Hae 1474

See text for details of the construction. The key for the various elements is as in Figure 10.

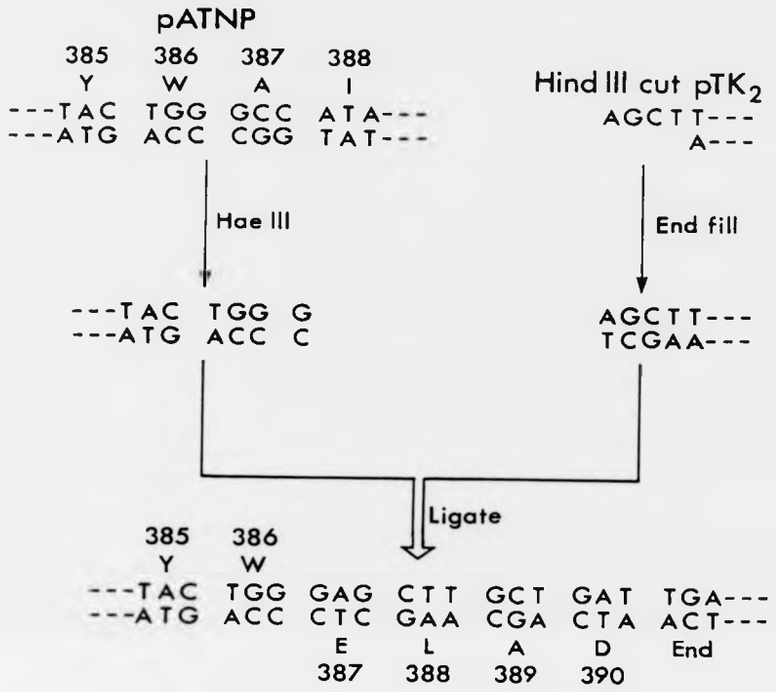


Figure 19 Construction of the plasmid pTKNP Hae 1474 at the nucleotide level

The encoded amino acids and their positions in NP are shown at the relevant Hae III site in the plasmid pATNP. In the final construct the translation termination codon from the stop region is also shown.

3. Construction of mutant plasmids lacking regions from within the NP gene

By using carefully chosen restriction enzymes it is possible to remove a region from within the NP coding sequence of pTKNP such that religation of the remaining amino and carboxyl termini recreates the correct reading frame. Four clones were constructed using this procedure:

a) Construction of the plasmid pTKNP Bam 577/793

The plasmid pTKNP contains four Bam Hl sites; two within the NP coding region (at positions 577 and 793); one in the pAT/Pvu II/8 sequence at the carboxyl end of the NP gene (1839) and one in the SV40 region (2376). A Bam Hl partial digestion of pTKNP was performed and the 5207 bp fragment from Bam Hl (793) to Bam Hl (577) was purified by gel electrophoresis. This was ligated to itself to give pTKNP Bam 577/793 (Figure 20). The relevant manipulations are shown at the nucleotide level in Figure 21.

The predicted protein from pTKNP Bam 577/793 has a MW of 47,512 and is identical to NP apart from lacking amino acids 88 to 159 inclusive.

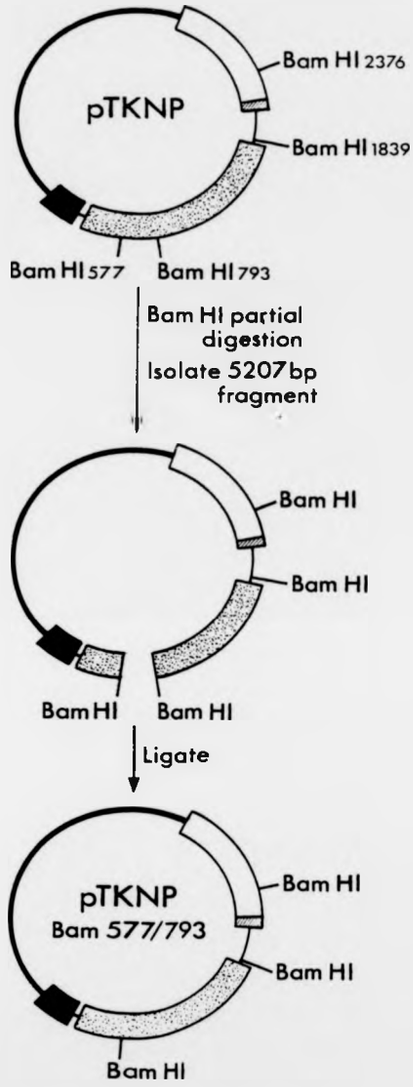


Figure 20 Construction of the plasmid pTKNP Bam 577/793

See text for details of the construction. The key for the various elements is as in Figure 10.

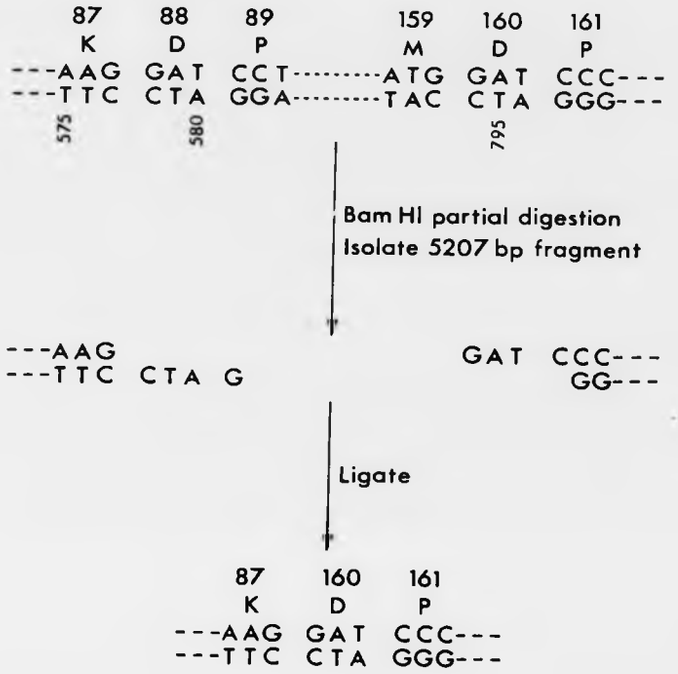


Figure 21 Construction of the plasmid pTKNP Bam 577/793 at the nucleotide level

Only those regions around the Bam HI sites at positions 577 and 793 are shown. The numbers below the sequence refer to the positions of the nucleotides in the plasmid pTKNP while above the sequence are the encoded amino acids and their positions in NP.

b) Construction of the plasmid pTKNP Pvu 705/1011

The plasmid pTKNP contains four Pvu II sites; the one used as the reference origin (position -3) and three within the NP coding region (at positions 705, 1011 and 1295). A Pvu II partial digestion of pTKNP was performed and the 5117 bp fragment from Pvu II (1011) to Pvu II (705) was purified by gel electrophoresis. This was ligated to itself to give pTKNP Pvu 705/1011 (Figure 22). The relevant manipulations are shown at the nucleotide level in Figure 23.

The predicted protein from pTKNP Pvu 705/1011 has a MW of 44,357 and is identical to NP apart from lacking amino acids 131 to 232 inclusive.

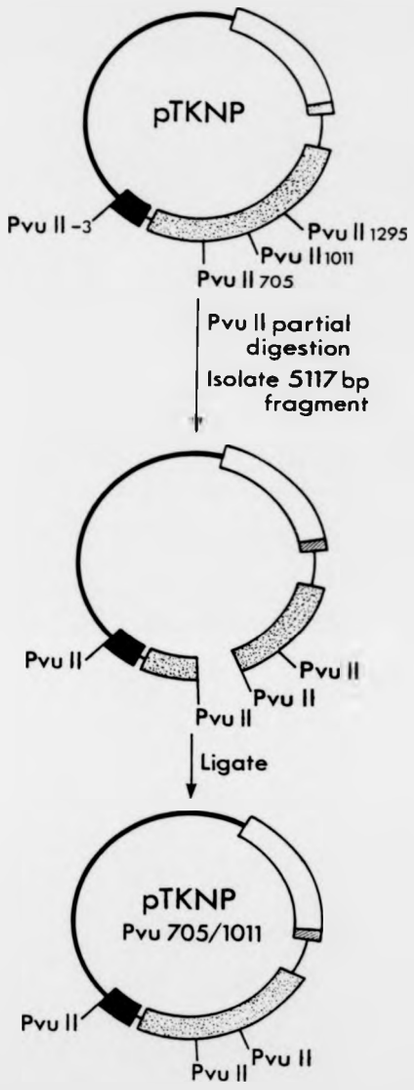


Figure 22 Construction of the plasmid pTKNP Pvu 705/1011

See text for details of the construction. The key for the various elements is as in Figure 10.

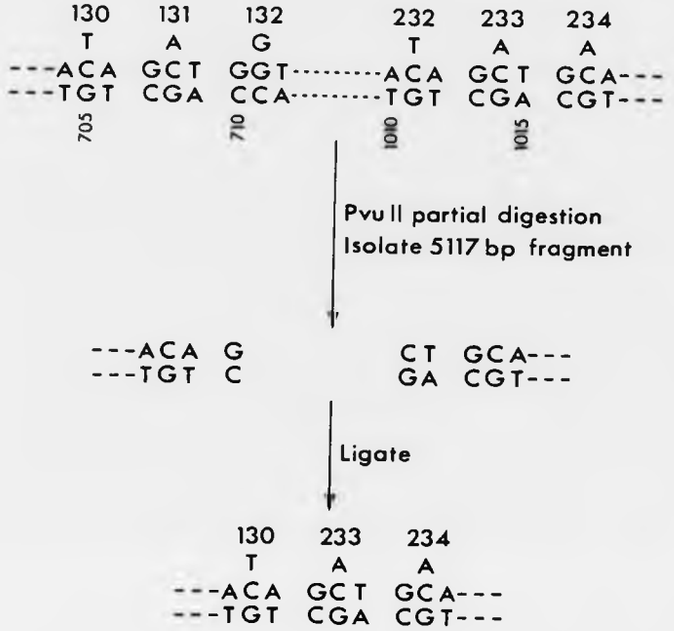


Figure 23 Construction of the plasmid pTKNP Pvu 705/1011 at the nucleotide level

Only those regions around the Pvu II sites at positions 705 and 1011 are shown. The numbers below the sequence refer to the positions of the nucleotides in the plasmid pTKNP while above the sequence are the encoded amino acids and their positions in NP.

c) Construction of the plasmid pTKNP Bgl 1078/1333

The plasmid pTKNP contains two Bgl II sites, both in the NP coding region (at positions 1078 and 1333). A Bgl II digestion of pTKNP was performed and the 5168 bp fragment from Bgl II (1333) to Bgl II (1078) was purified by gel electrophoresis. This was ligated to itself to give pTKNP Bgl 1078/133 (Figure 24). The relevant manipulations are shown at the nucleotide level in Figure 25.

The predicted protein from pTKNP Bgl 1078/1333 has a MW of 46,630 and is identical to NP apart from lacking amino acids 255 to 339 inclusive.

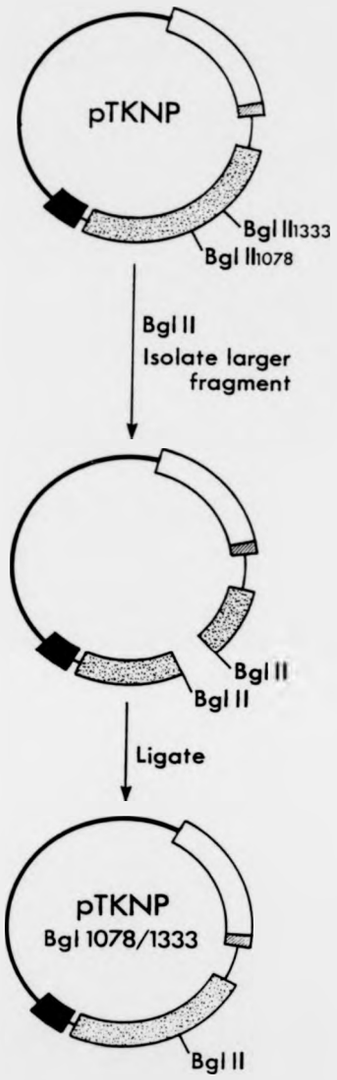


Figure 24 Construction of the plasmid pTKNP Bgl 1078/1333

See text for details of the construction. The key for the various elements is as in Figure 10.

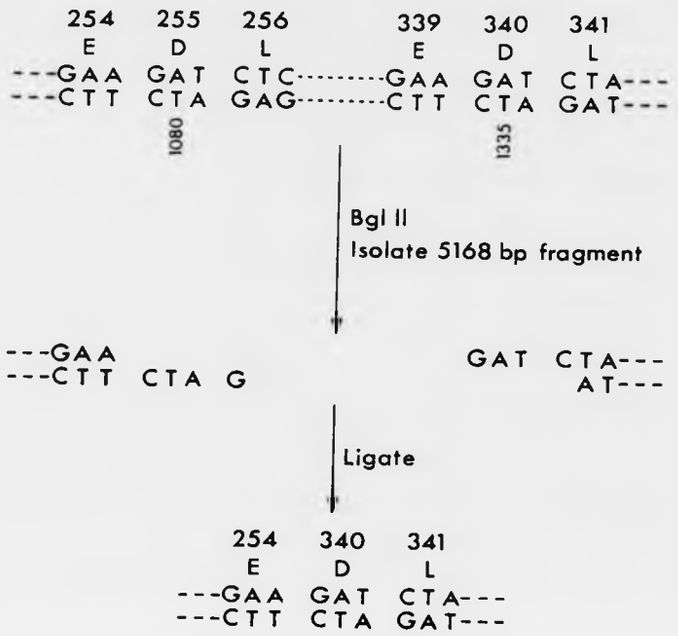


Figure 25 Construction of the plasmid pTKNP Bgl 1078/1333 at the nucleotide level

Only those regions around the Bgl II sites at positions 1078 and 1333 are shown. The numbers below the sequence refer to the positions of the nucleotides in the plasmid pTKNP while above the sequence are the encoded amino acids and their positions in NP.

d) Construction of the plasmid pTKNP IMP 1295

The plasmid pTKNP has seven Aha II sites. The first of these, in the direction of transcription, is at position 319 which is just downstream of the NP initiation codon. Digestion of pTKNP with Pvu II and Aha II produced a 322 bp fragment containing the TK promotor and the ATG initiator. This was purified, end filled and ligated to the 4125 bp fragment (from Pvu II (1295) to Pvu II (-3)) produced by Pvu II digestion of pTKNP. These manipulations are illustrated in Figure 26 and at the nucleotide level in Figure 27. The resulting construct, pTKNP IMP 1295, should direct the synthesis of a protein having a MW of 19,164 and be identical to NP apart from lacking amino acids 3 to 327 inclusive.

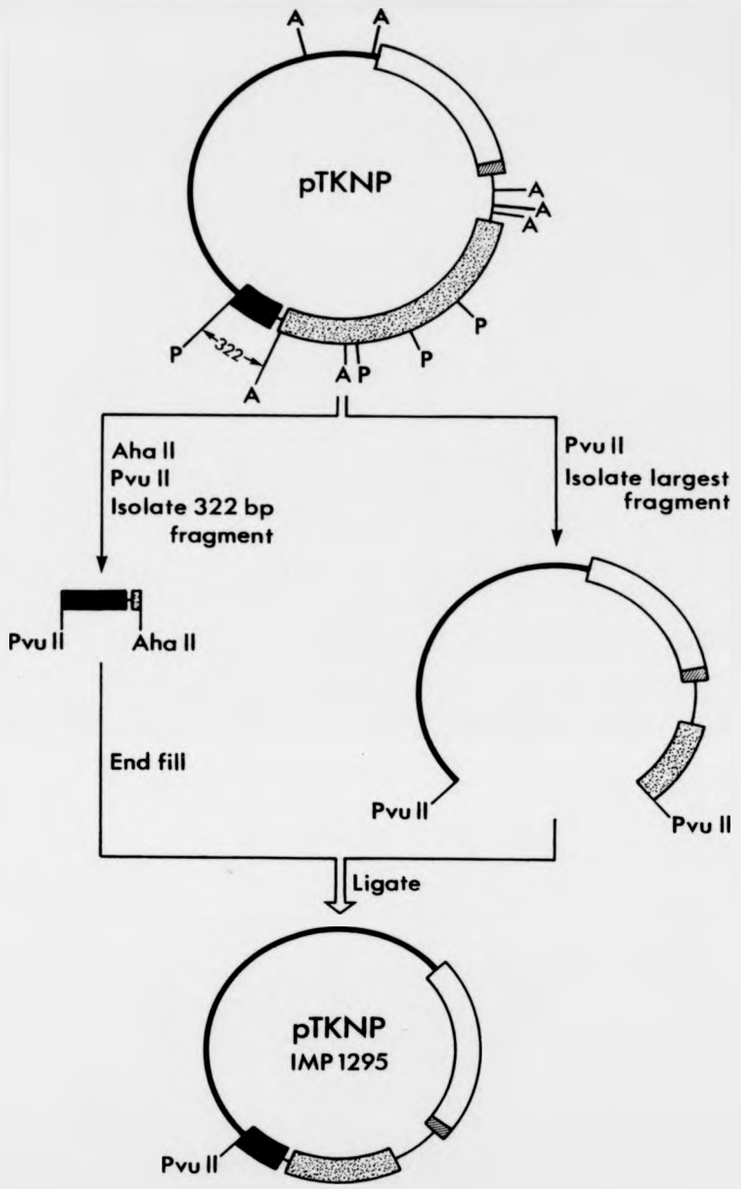


Figure 26 Construction of the plasmid pTKNP IMP 1295

See text for details of the construction. The key for the various elements is as in Figure 10. P represents Pvu II sites and A represents AHA II sites.

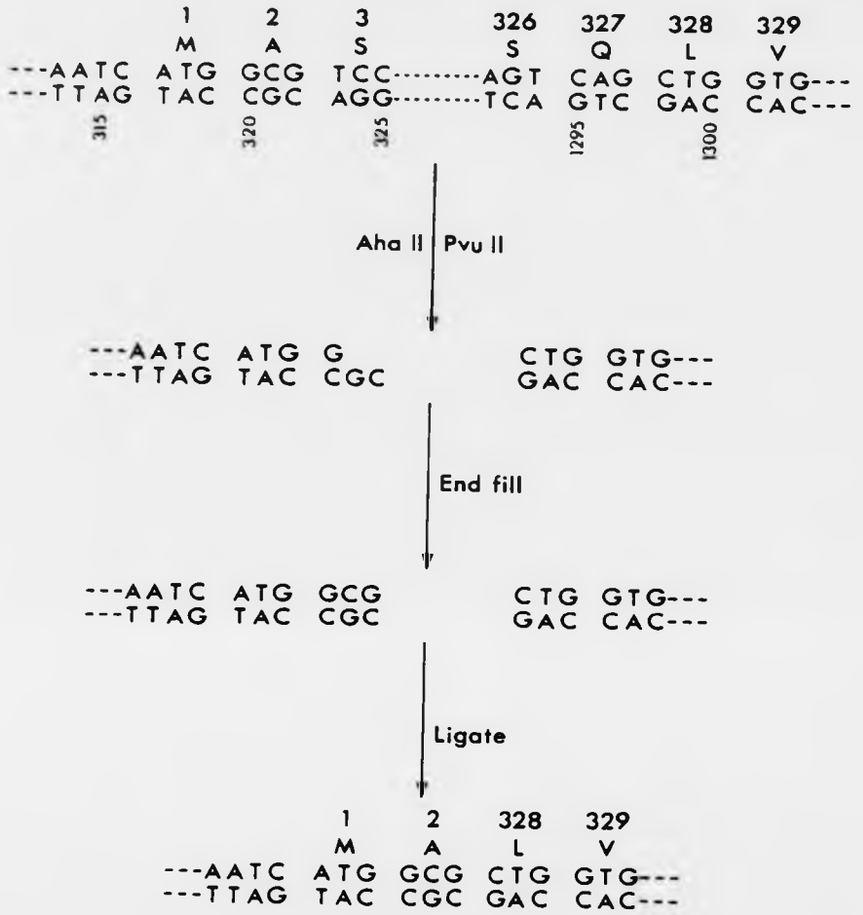


Figure 27 Construction of the plasmid pTKNP IMP 1295 at the nucleotide level

Only those regions around the Aha II sites at position 319 and the Pvu II site at 1295 are shown. The numbers below the sequence refer to the position of the nucleotides in the plasmid pTKNP while above the sequence is the encoded amino acid and its position in NP.

4. Construction of mutant plasmids lacking various amounts of the amino terminus of the NP gene

In the construction of these mutants use was made of the Hind III site at position 263 which is in the short pAT/Pvu II/8 sequence at the amino end of the NP gene and is therefore after the TK transcription promotor region but before the authentic NP translation initiation codon. The idea was to remove from pTKNP a region from a chosen restriction site within the NP coding sequence to this Hind III site and thereby remove that region of NP to the amino side of the chosen site. Five clones were constructed using this procedure:

a) Construction of the plasmid pTKNP Hind 1348C

The plasmid pTKNP contains three Hind III sites; one in the short pAT/Pvu II/8 sequence at the amino end of the NP gene (position 263); one within the NP coding region (1348) and a third which is recreated at the carboxyl end of the NP insert (2118). A Hind III partial digestion of pTKNP was performed and the 4338 bp fragment from Hind III (1348) to Hind III (263) was purified by gel electrophoresis. This was ligated to itself to give pTKNP Hind 1348C. These manipulations are illustrated diagrammatically in Figure 28 while Figure 29 shows the relevant manipulations at the nucleotide level.

The first ATG after the transcriptional promotor in pTKNP Hind 1348C spans the codons that encoded amino acids 368 and 369 in the authentic NP and so translation initation at this point will give a protein bearing no resemblance to NP. In fact, initiation at this triplet gives a peptide of 13 amino acids (Figure 30). Translation initiation at the second ATG, which encodes amino acid number 371 in the authentic NP, would give a protein having the last 128 amino acids of NP and a MW of 14,262.

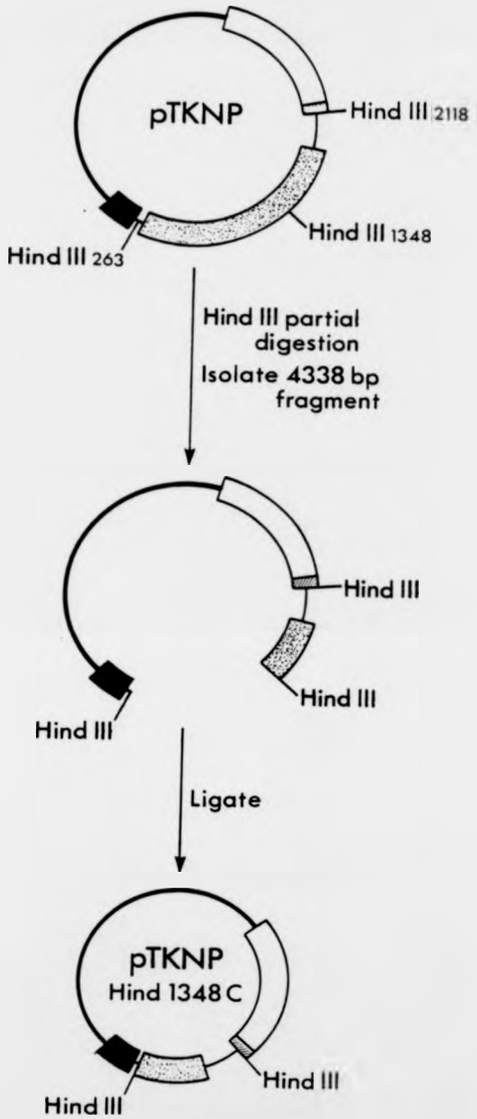


Figure 28 Construction of the plasmid pTKNP Hind 1348C

See text for details of the construction. The key for the various elements is as in Figure 10.

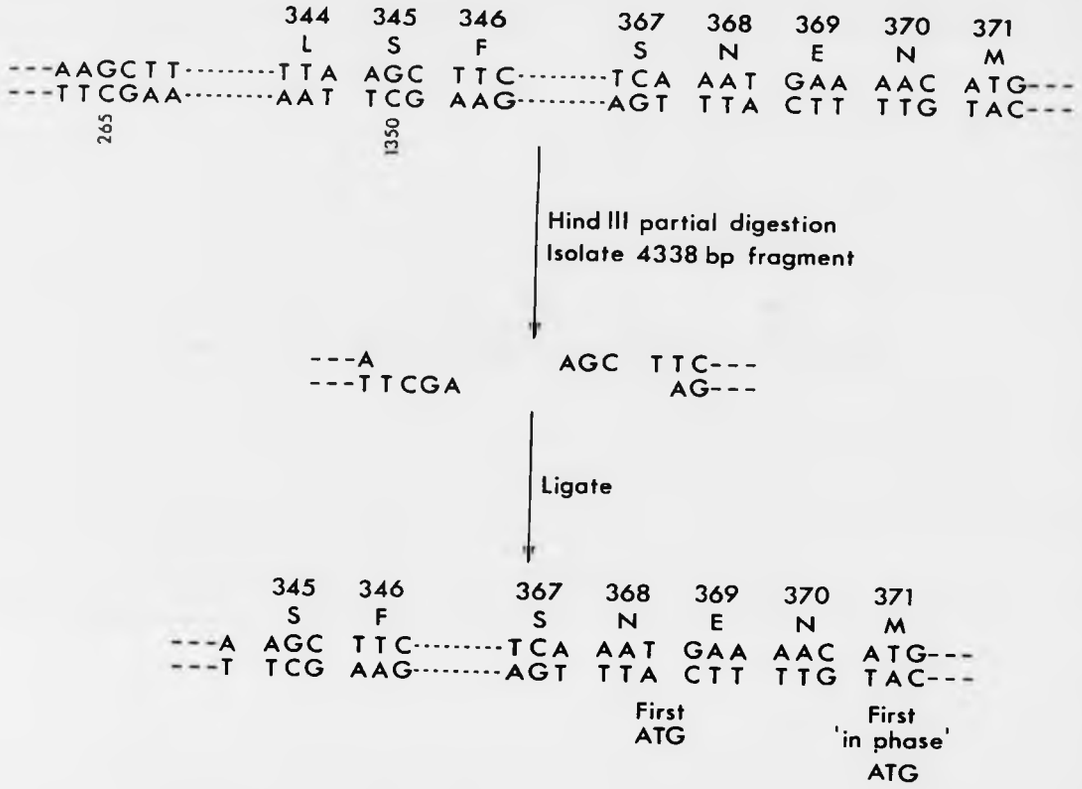


Figure 29 Construction of the plasmid pTKNP Hind 1348C at the nucleotide level

Only those regions around the Hind III sites at positions 263 and 1348 and at the first ATG codon after 1348 are shown. The numbers below the sequence refer to the positions of the nucleotides in the plasmid pTKNP while above the sequence are the encoded amino acids and their positions in NP. The final construct shows the first downstream ATG and the first in phase ATG.

Figure 30 The peptide resulting from translation initiation at the first ATG in pTKNP Hind 1348C

The nucleotide sequence is written in the coding sense. Above the sequence is the encoded amino acid and its position in NP while below the sequence is the peptide resulting from initiation at the first ATG in pTKNP Hind 1348C.

b) Construction of the plasmids pTKNP Met 136, pTKNP Met 238 and pTKNP Met 331/S

The plasmid pTKNP contains four Pvu II sites; the one used as the reference origin (-3) and three within the NP coding region (at positions 705, 1011 and 1295). After partial digestion with Pvu II three DNA fragments were purified by electrophoresis; the 4715 bp fragment from Pvu II (705) to Pvu II (-3); the 4409 bp fragment from Pvu II (1011) to Pvu (-3) and the 4125 bp fragment from Pvu II (1295) to Pvu II (-3). These fragments were ligated to the 266 bp fragment containing the TK promotor region that was isolated after digestion of pTKNP with Pvu II and Hind III. This fragment was end filled prior to ligation. Ligation gave the plasmids pTKNP Met 136, pTKNP Met 238 and PTKNP Met 331/S respectively. These manipulations are illustrated in Figure 31.

In pTKNP Met 136 it is thought that translation initiation will occur at the ATG codon encoding amino acid number 136 in NP (Figure 32). This would give a product containing the last 363 amino acids of NP and having a MW of 40,407. In pTKNP Met 238 it is thought that translation initiation will occur at the ATG codon encoding amino acid number 238 in NP (Figure 33). This would give a product containing the last 261 amino acids of NP and having a MW of 28,856. In pTKNP Met 331/S it is thought that translation initiation will occur at the ATG codon encoding amino acid number 331 in NP (Figure 34). This would give a product containing the last 168 amino acids of NP and having a MW of 18,564.

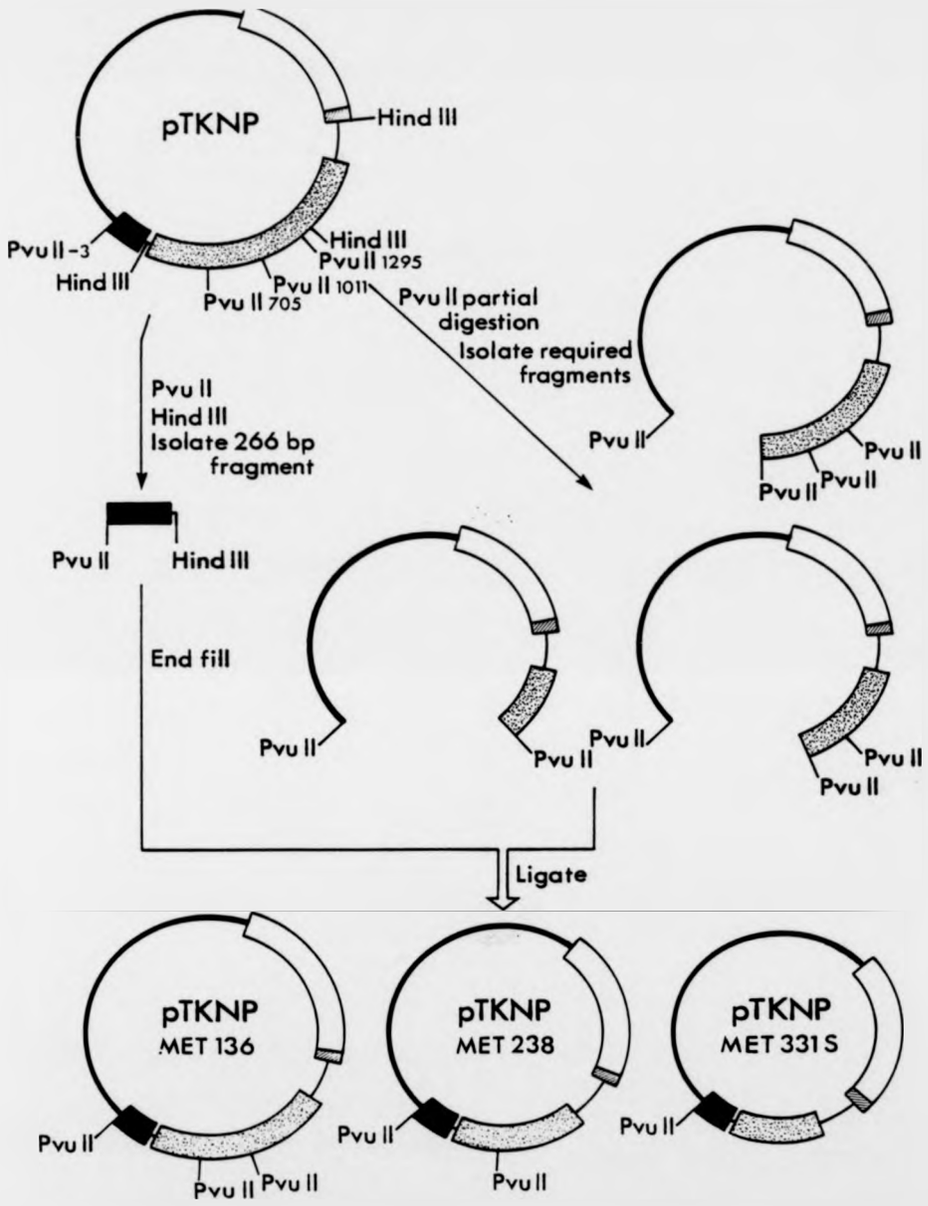


Figure 31 Construction of the plasmids pTKNP Met 136, pTKNP Met 238 and pTKNP Met 331/S

See text for details of the construction. The key for the various elements is as in Figure 10.

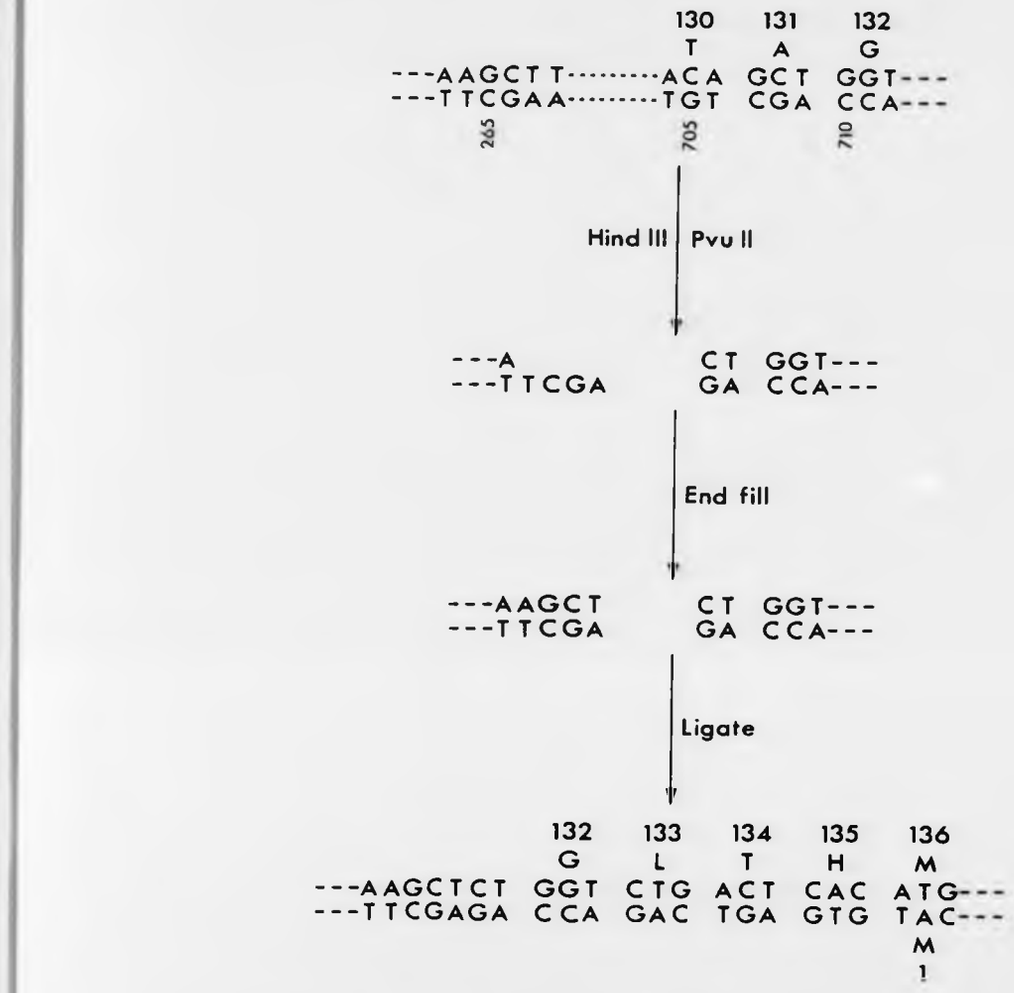


Figure 32 Construction of the plasmid pTKNP Met 136 at the nucleotide level

Only those regions around the Hind III sites at position 263 and and the Pvu II site at 705 are shown. Above the sequence is the encoded amino acid and its position in NP while below the sequence is initially the position of the nucleotides in pTKNP, and finally the position of proposed translation initiation in the mutant construct.

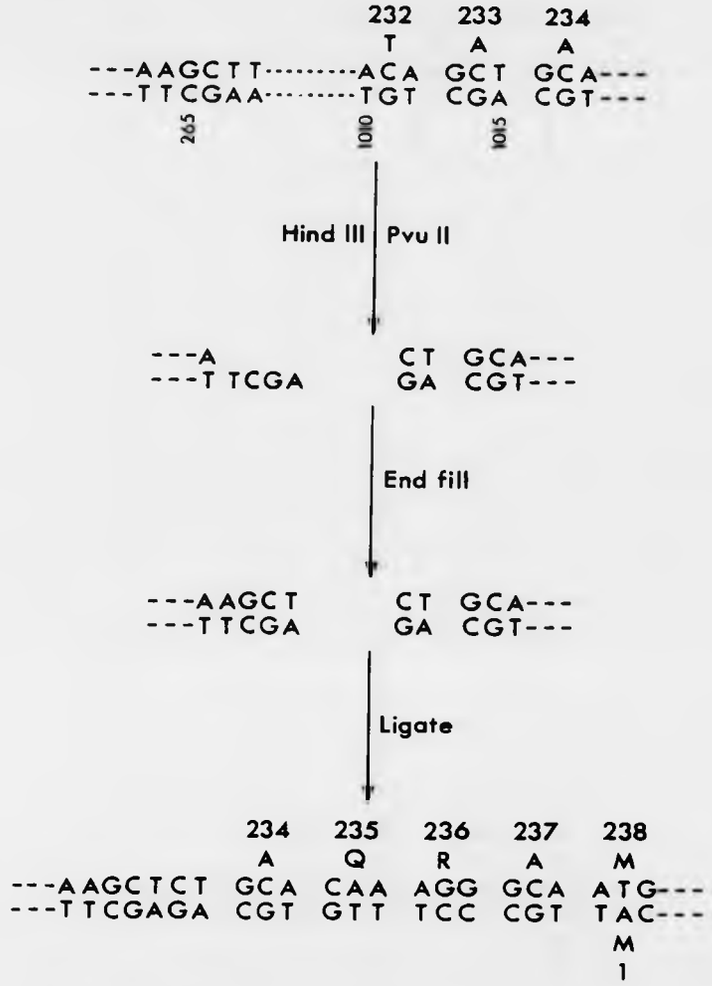


Figure 33 Construction of the plasmid pTKNP Met 238 at the nucleotide level

Only those regions around the Hind III sites at position 263 and the Pvu II site at 1011 are shown. Above the sequence is the encoded amino acid and its position in NP while below the sequence is initially the position of the nucleotides in pTKNP, and finally the position of proposed translation initiation in the mutant construct.

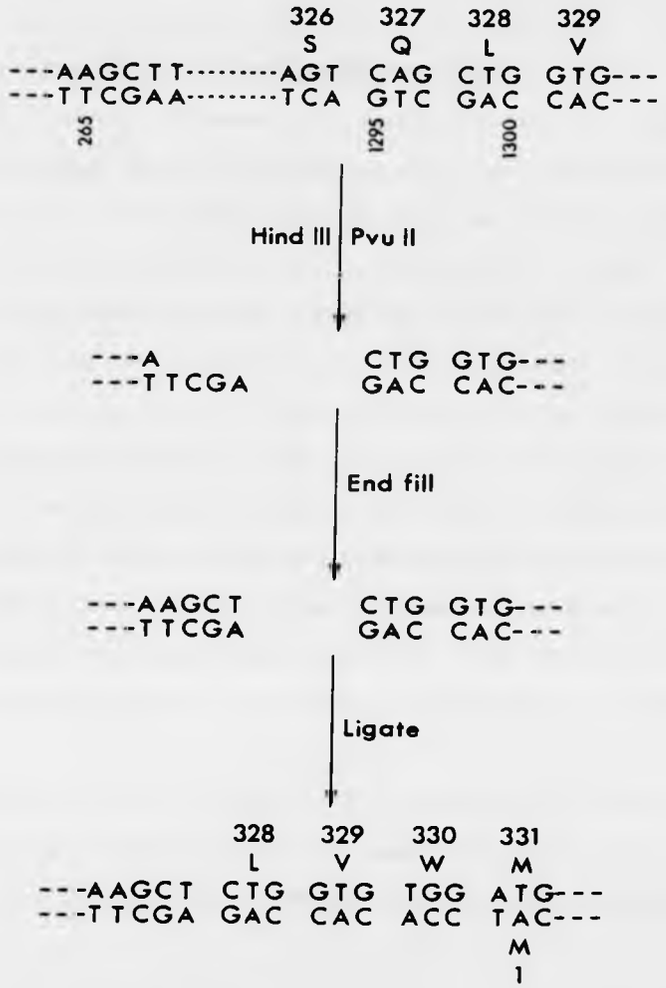


Figure 34 Construction of the plasmid pTKNP Met 331/S at the nucleotide level

Only those regions around the Hind III sites at position 263 and the Pvu II site at 1295 are shown. Above the sequence is the encoded amino acid and its position in NP while below the sequence is initially the position of the nucleotides in pTKNP, and finally the position of proposed translation initiation in the mutant construct.

c) Construction of the plasmid pTKNP Met 331/L

Due to the presence of inconvenient restriction sites in pTKNP the plasmid pATNP was used as the starting point in the construction of pTKNP Met 331/L. The plasmid pATNP has two Acc I sites: one within the NP gene and one downstream of the carboxyl end of the gene. This second site is also recognised by the enzyme Sal I although this pair of isoschizomers cut the target site at different places. Digestion of pATNP with Sal I produced a linear molecule which was purified and further digested with Acc I. The smaller of the two fragments produced by this second digestion (the 864 bp Acc I/Sal I fragment of pATNP) was purified, end filled and blunt end ligated into the end filled Hind III site of pTKNP. The resulting clone is identical to pTKNP at the carboxyl end of the insert (see Figure 9). These manipulations are illustrated in Figure 35, and at the nucleotide level in Figure 36.

In pTKNP Met 331/L it is thought that translation initiation will occur at the ATG codon encoding amino acid number 331 in NP. This would give a product containing the last 168 amino acids of NP and having a MW of 18,564.

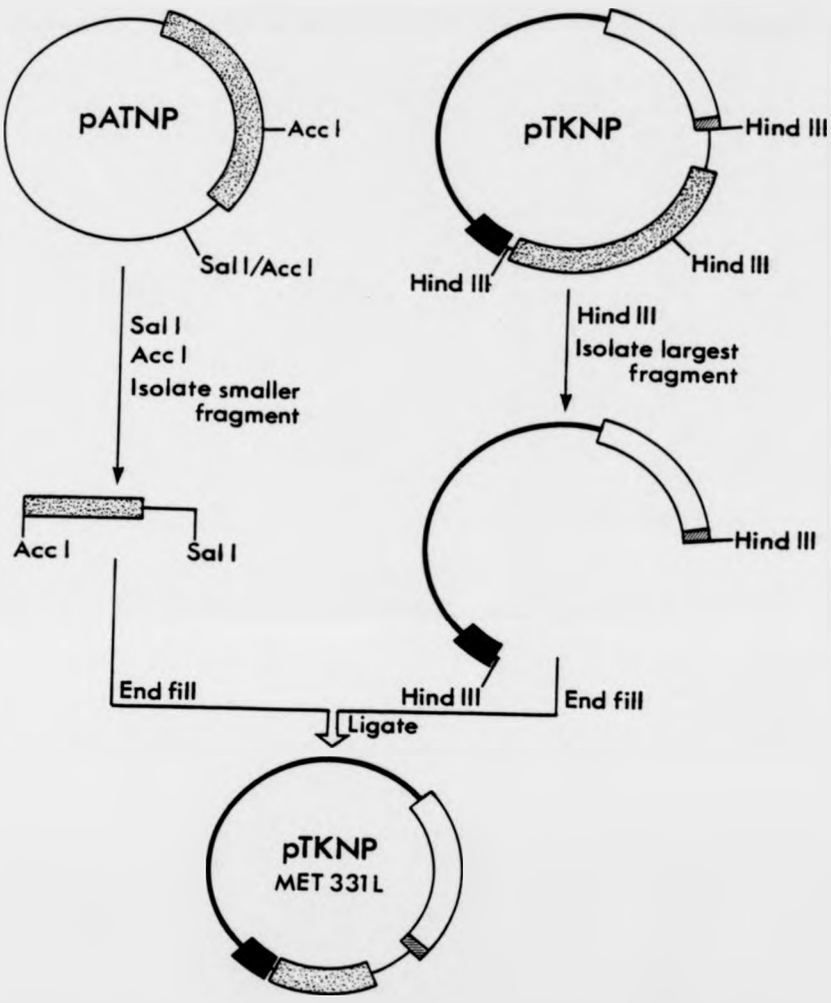


Figure 35 Construction of the plasmid pTKNP Met 331/L

See text for details of the construction. The key for the various elements is as in Figure 10.

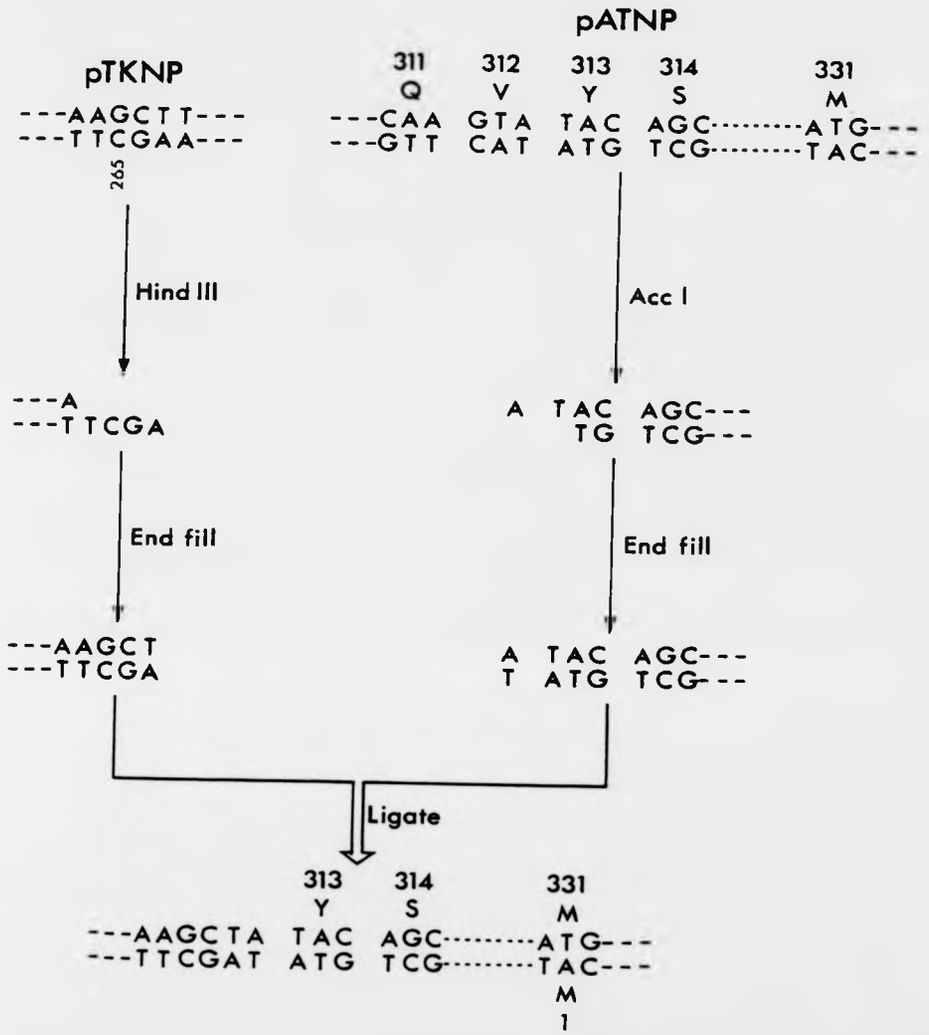


Figure 36 Construction of the plasmid pTKNP Met 331/L at the nucleotide level

The regions around the Hind III site at position 263 in pTKNP and the relevant Acc I site in pATNP are shown. Also shown is the first ATG downstream of this Acc I site. Above the bottom sequence is the encoded amino acid and its position in NP while below the sequence is the position of the proposed translation initiation in the mutant construct.

5. Construction of the plasmid pTKG

The plasmid $pGOV_2$ (kindly supplied by V. Lingappa, UCSF California, USA) contains a full length cDNA copy of the chimpanzee $\alpha_1$globin. At the carboxyl end of the globin coding region there is part of a cDNA copy of chicken ovalbumin from the Pst I site at amino acid 139 through to the translation terminator. Digestion of $pGOV_2$ with Nco I (the Nco I site spans the initiation codon of the globin gene) and Stu I (the Stu I site spans the codon for amino acid number 252 of the ovalbumin gene) yielded a fragment of 990 bp which was purified by electrophoresis.

The plasmid pTK_2OV+ (kindly supplied by A. Colman; Krieg et al., 1984) contains a full length cDNA copy of the chicken ovalbumin gene cloned into the Hind III site of the expression vector pTK_2. Digestion of pTK_2OV+ with Nco I (the Nco I site spans the initiation codon of the ovalbumin gene) and Stu I (the Stu I site spans the codon for amino acid number 252 of the ovalbumin gene) yielded a large fragment of 4121 bp which was purified by electrophoresis.

The two fragments were ligated to give the plasmid pTKG which has the complete globin gene followed by part of the chicken ovalbumin gene from the Pst I site at amino acid 139 through to the translation terminator (amino acids 139 to 251 are encoded by sequences from the plasmid $pGOV_2$ and amino acids 252 to the end being encoded by sequences from the plasmid pTK_2OV+). It should be noted that the short sequence upstream of the globin initator in pTKG is derived from the ovalbumin gene. These manipulations are illustrated in Figure 37.

The plasmid pTKG should direct the synthesis of wild-type chimpanzee $\alpha_1$globin since the coding region is intact and the initiator codon is the first ATG downstream of the TK transcription promoter.

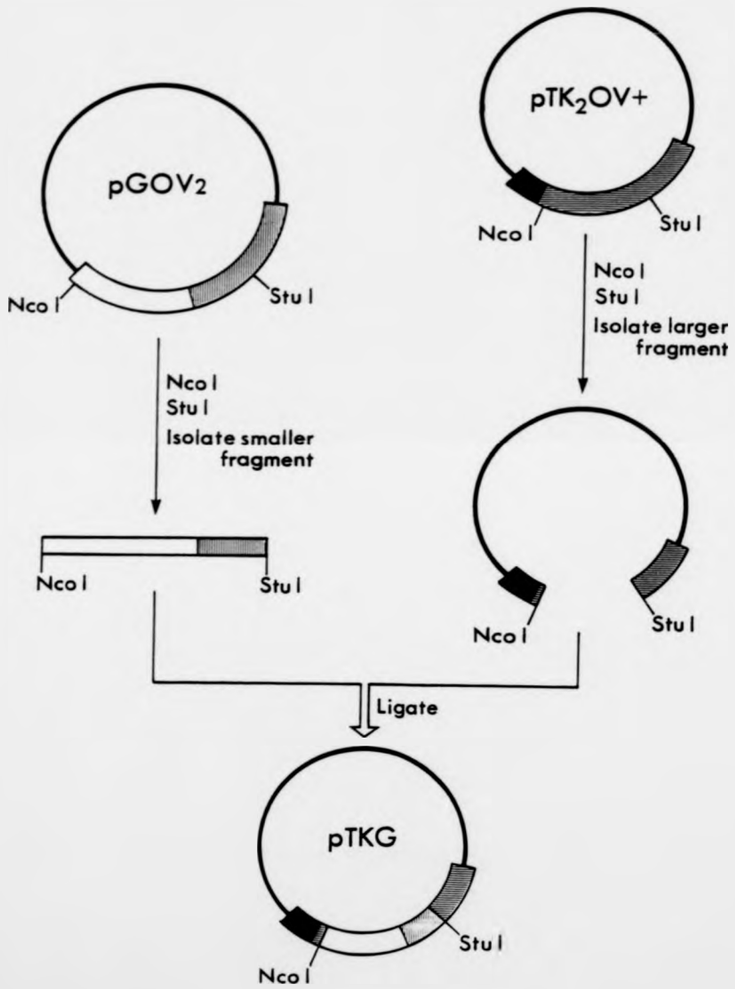


Figure 37 Construction of the plasmid pTKG

See text for details of the construction. The only elements shown are the globin gene (open box), the ovalbumin gene (hatched box) and the TK promotor region (solid box). The cross-hatching of the ovalbumin gene is done at different angles to indicate from which parent plasmid the various regions were derived.

6. Construction of plasmids encoding NP-globin fusion proteins

The plasmid pTKG has unique Nco I and Pst I sites. The Nco I site spans the globin initiation codon while the Pst I site is at the end of the globin cDNA, so Nco I/Pst I digestion of pTKG will remove the globin gene in its entirity. The plasmid pTKG was cut with Nco I, end filled, cut with Pst I and the 650 bp fragment containing the globin gene purified by electrophoresis. This was inserted into M13 mp10 which had been cut with Bam HI and Pst I and had had the Bam HI site end filled. The resulting clone, M13G, was digested with Sma I and Bgl I to release a 500 bp fragment containing the entire globin coding region (the Bgl I site is downstream of the globin termination codon). This is called fragment A. In the literature M13 mp10 has a Bgl I site but I was unable to cut the DNA at this site.

There are two Bgl I sites in pTKG; one downstream of the globin termination codon and the other is in the β-lactamase gene of the vector region. Digestion of pTKG with Bgl I yields a 2660 bp fragment. This is called fragment B.

These manipulations are illustrated in Figure 38 while Figure 39 shows how these manipulations affect the region around the globin initiation codon.

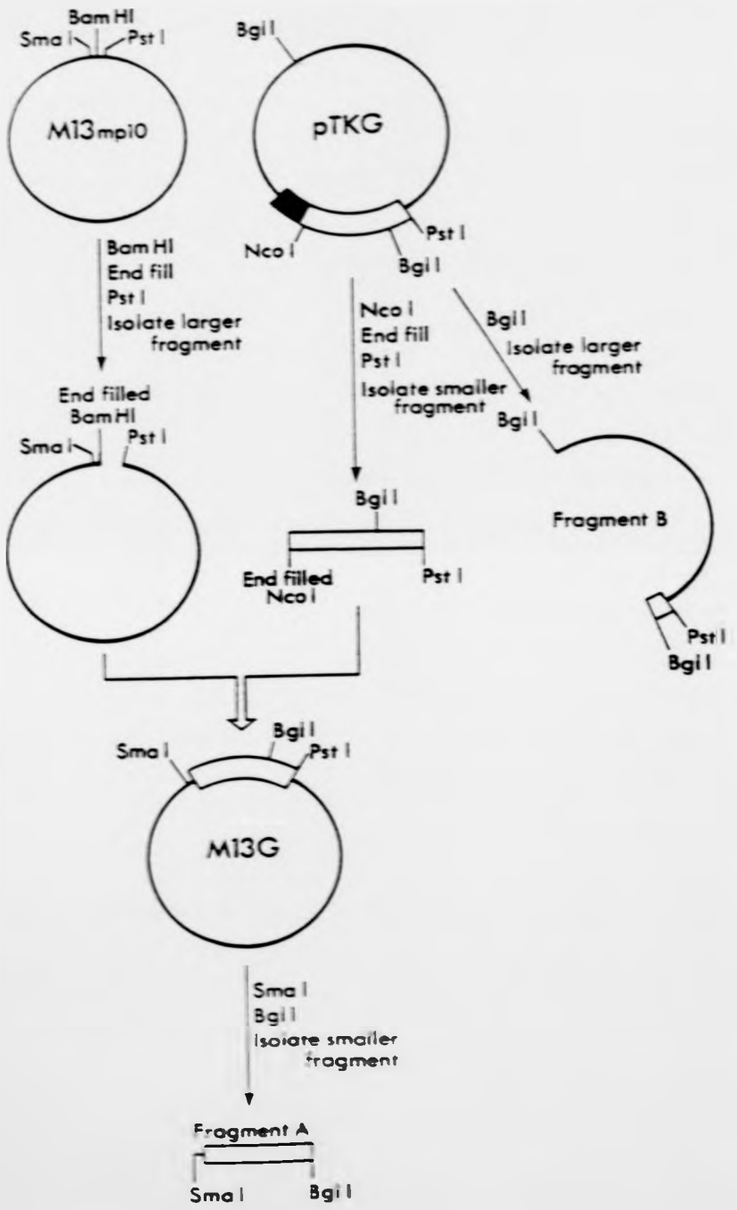


Figure 38 Construction of the Fragments A and B

See text for details of the construction. For simplification the plasmids are not drawn to scale and the majority of elements in pTKG are omitted, only the TK transcriptional promotor region (solid box) is shown to indicate the direction of transcription. The globin cDNA is also shown (open box).

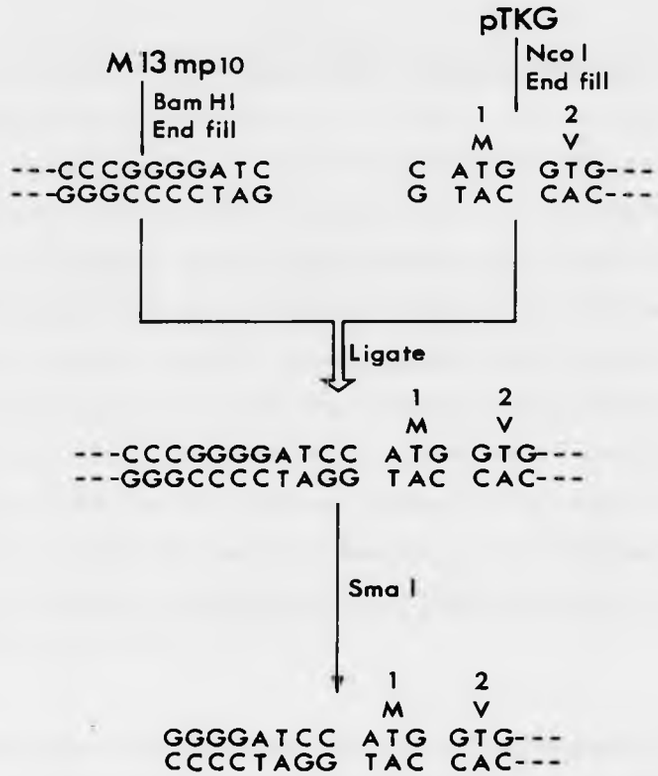


Figure 39 Construction of Fragment A at the nucleotide level

This shows how the construction of Fragment A affects the region around the globin initiation codon. The first two amino acids of globin are shown.

a) Construction of the plasmids pTKNP 130G and pTKNP 232G

The plasmid pTKNP contains four Pvu II sites: the one used as the reference origin (position -3) and three within the NP coding region, at positions 705, 1011 and 1295. Partial digestion of pTKNP with Pvu II was followed by Bgl I digestion and purification of two fragments: the 2379 bp fragment from Bgl I to Pvu II (705) and the 2685 bp fragment from Bgl I to Pvu II (1011). These fragments were ligated to fragments A and B (see Figure 38) to give the plasmids pTKNP 130G and pTKNP 232G respectively. These manipulations are illustrated in Figure 40. It should be pointed out that although fragment B can insert in either direction, the fact tht the Bgl I site is in the β-lactamase gene means that the "incorrect" orientation is not viable when grown in the presence of ampicillin.

The protein predicted from pTKNP 130G has 275 amino acids; the first 130 of NP, 3 encoded by the linking sequence originally from M13 mp10 and the 142 amino acids of globin (see Figure 41). The protein predicted from pTKNP 232G has 377 amino acids; the first 232 of NP, 3 encoded by the linking sequence and the 142 of globin (see Figure 42).

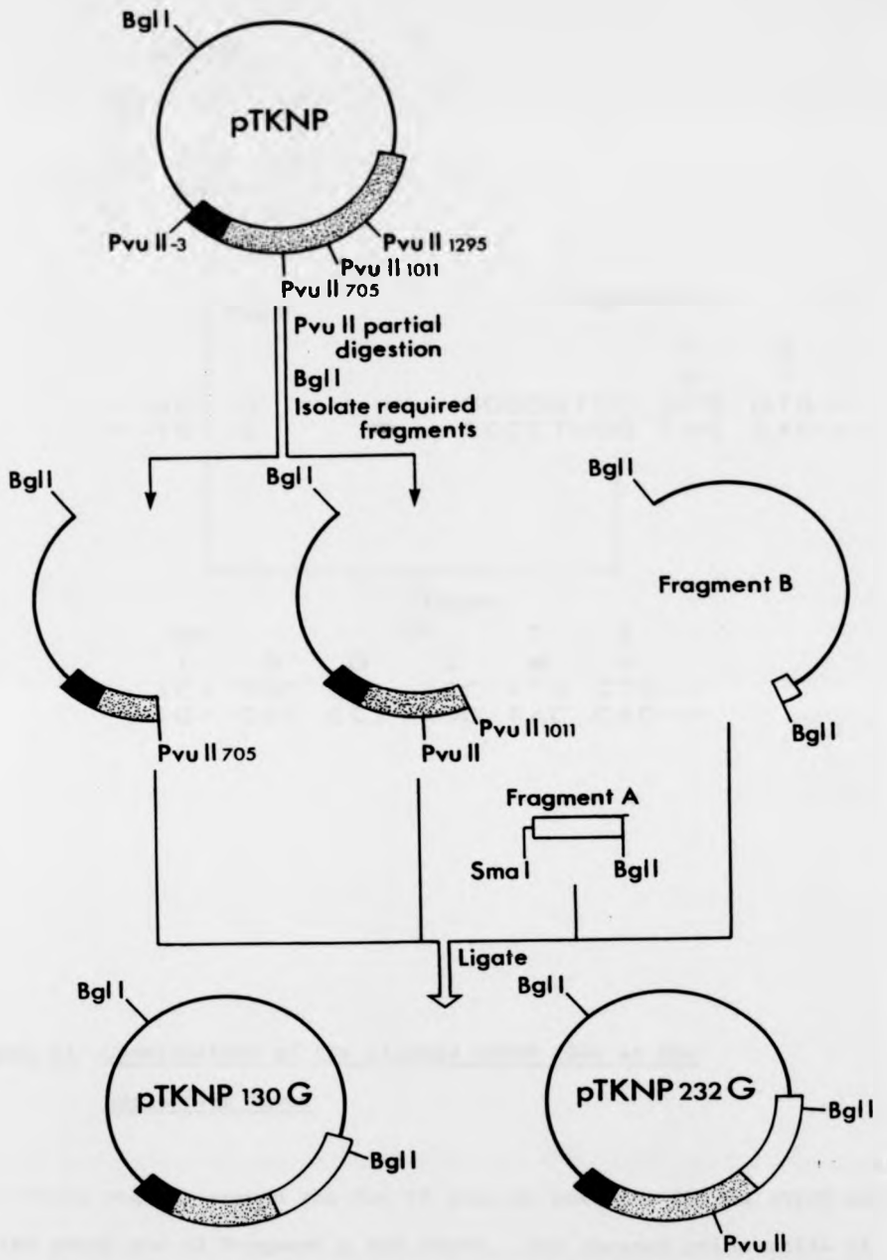


Figure 40 Construction of the plasmids pTKNP 130G and pTKNP 232G

See text for details of the construction. Only the TK transcription promotor (shaded box), the NP gene (stipled box) and the globin gene (open box) are shown. Fragments A and B are from Figure 38.

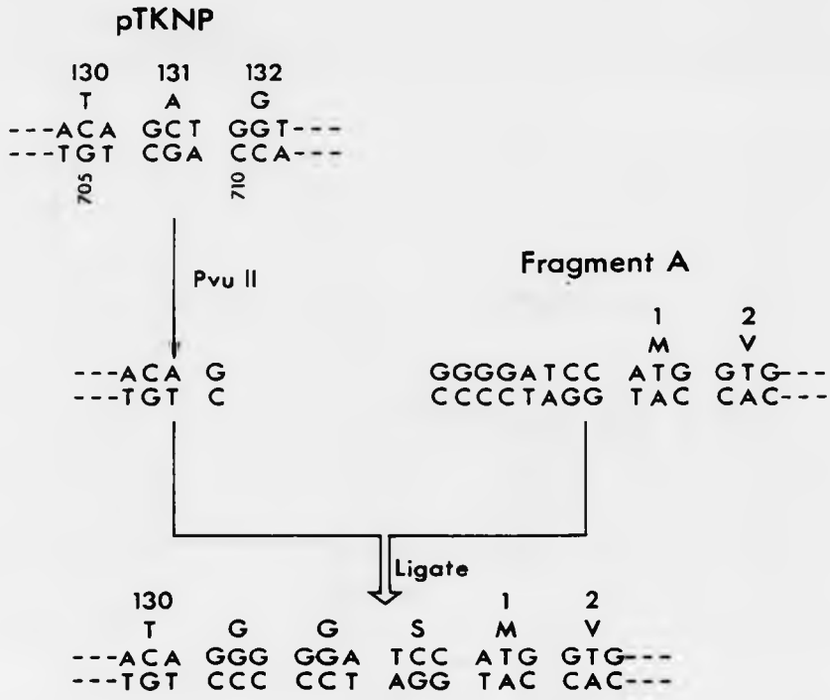


Figure 41 Construction of the plasmid pTKNP 130G at the nucleotide level

Only those regions around the Pvu II site at position 705 in pTKNP and at the amino end of Fragment A are shown. The encoded amino acids of NP, globin and the resulting fusion protein are shown above the sequence. The numbers above the amino acids indicate their positions in the wild-type proteins.

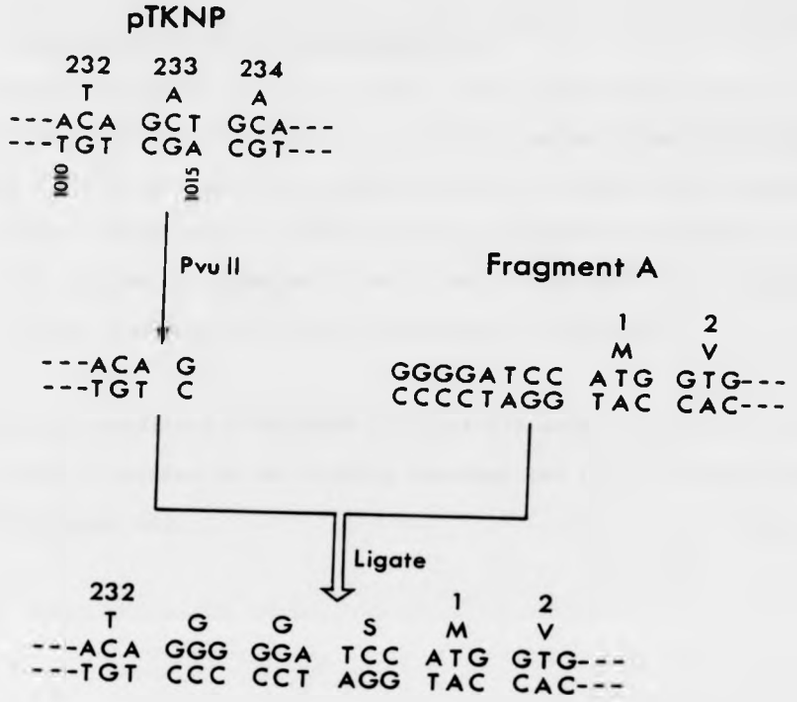


Figure 42 Construction of the plasmid pTKNP 232G at the nucleotide level

Only those regions around the Pvu II site at position 1011 in pTKNP and at the amino end of Fragment A are shown. The encoded amino acids of NP, globin and the resulting fusion protein are shown above the sequence. The numbers above the amino acids indicate their positions in the wild-type proteins.

b) Construction of the plasmid pTKNP 312G

The plasmid pTKNP has two Acc I sites; one within the NP coding region (position 1250) and the other in the pBR322 region of the expression vector. pTKNP was partially digested with Acc I, end filled, digested with Bgl I and the 2673 bp fragment was purified by electrophoresis. This was ligated to fragments A and B (see Figure 38) to give pTKNP 312G. These manipulations are illustrated in Figure 43.

The protein predicted from pTKNP 312G has 457 amino acids; the first 312 of NP, 3 encoded by the linking sequence and the 142 amino acids of globin (Figure 44).

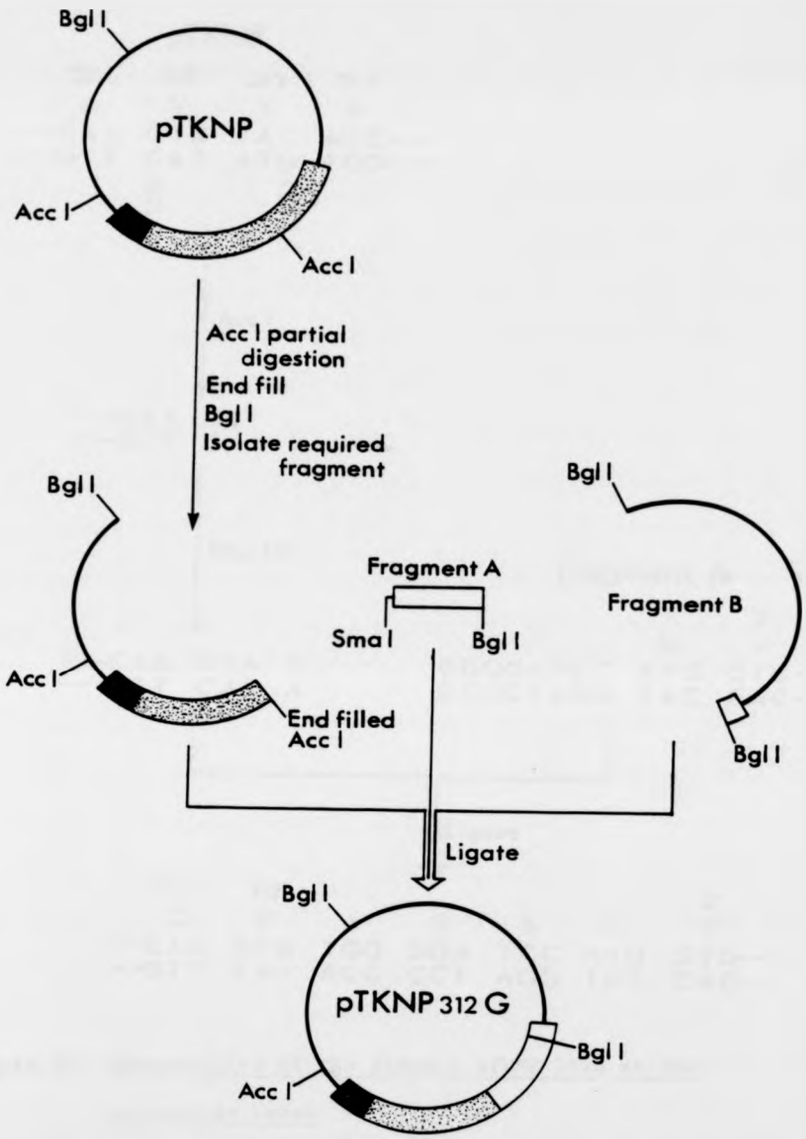


Figure 43 Construction of the plasmid pTKNP 312G

See text for details of the construction. Only the TK transcription promotor (solid box), the NP gene (stipled box) and the globin gene (open box) are shown. Fragments A and B are from Figure 38.

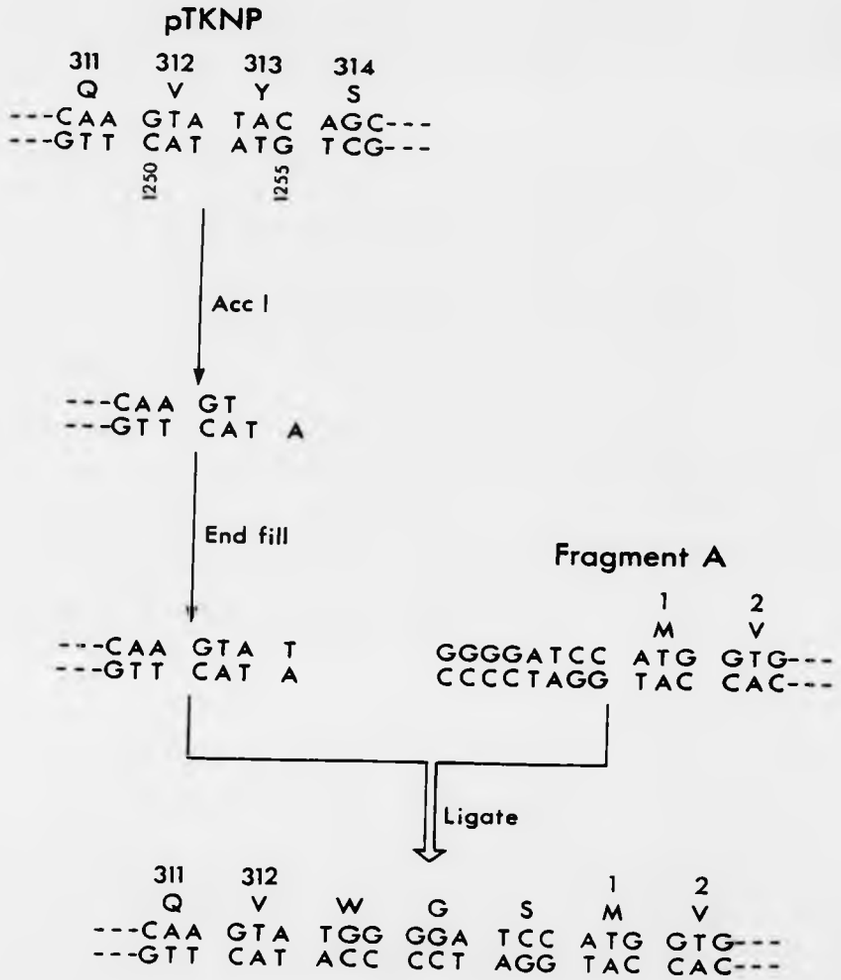


Figure 44 Construction of the plasmid pTKNP 312G at the nucleotide level

Only those regions around the Acc I site at position 1250 in pTKNP and at the amino end of Fragment A are shown. The encoded amino acids of NP, globin and the resulting fusion protein are shown above the sequence. The numbers above the amino acids indicate their positions in the wild-type proteins.

7. Construction of the plasmid pTKNPG

The plasmid pTKNP has a unique Nae I site at position 1865 which is downstream of the NP termination codon at position 1811. The enzyme Nae I was used to linearise pTKNP and this was treated with exonuclease Bal-31 to produce a series of molecules containing various amounts of the NP gene. These molecules were digested with Bgl I and ligated to a fragment obtained from pTKG. The plasmid pTKG contains two Bgl I sites and a unique Nco I site which spans the globin initiation codon. Digestion with Nco I was followed by an end filling reaction and partial digestion with Bgl I. The resulting 3160 bp fragment contains the complete globin gene. These manipulations are illustrated in Figure 45.

The clones were screened using restriction enzymes and a number were selected which had lost the NP termination codon. These were tested for production of a fusion protein in Xenopus oocytes. One clone, designated pTKNPG, produced a protein that reacted with antibodies to both NP and globin indicating that the proper reading frame was maintained between the NP and globin sequences. The protein had a MW of 65,000 which, allowing for approximately 12,500 MW of globin, suggests it contained the amino terminal 52,500 MW of NP. Restriction digestion of pTKNPG suggested that the Bal-31 treatment had removed the last 40 or so amino acids of NP which is consistent with the protein that was produced. The plasmid pTKNPG appears therefore to specify a chimeric protein with the first 460 amino acids of NP at its amino terminus and the entire globin sequence at its carboxyl terminus.

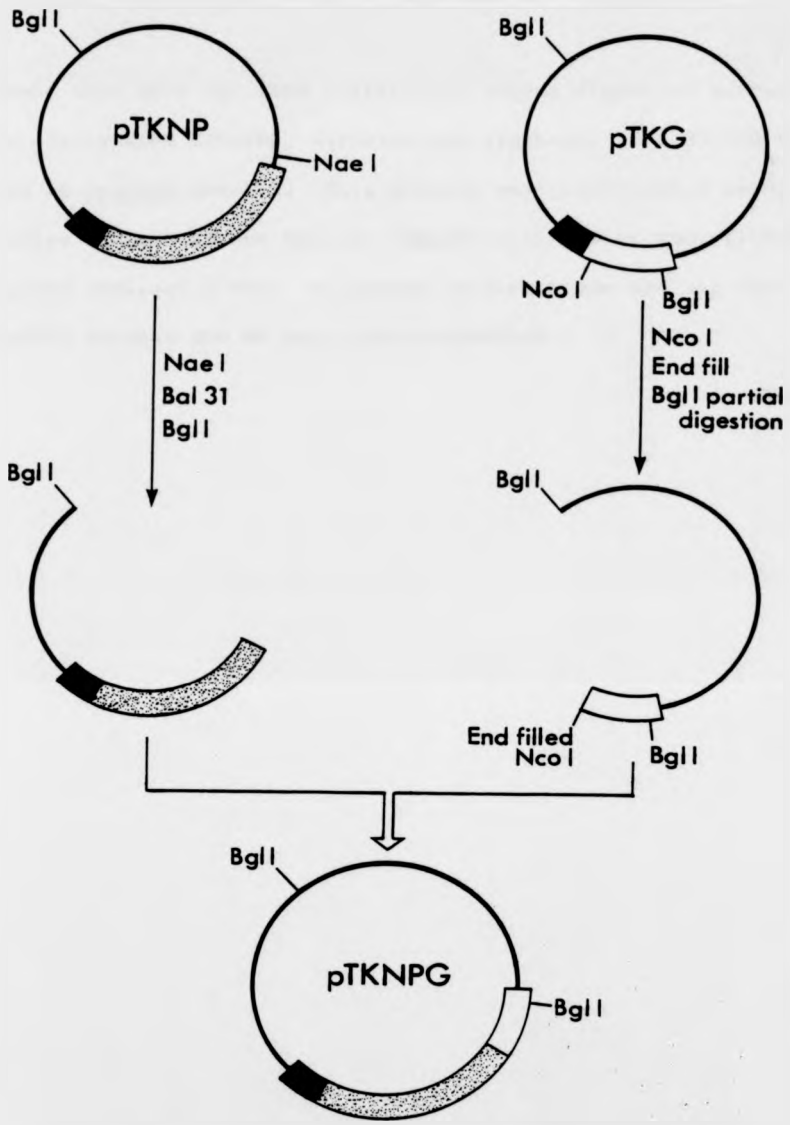


Figure 45 Construction of the plasmid pTKNPG

See text for details of the construction. Only the TK transcription promotor (solid box), the NP gene (stipled box) and the globin gene (open box) are shown.

A plasmid that gave the same restriction enzyme digestion pattern as pTKNPG, designated pTKNPMG, directed the synthesis of a 53,500 MW protein in Xenopus oocytes. This protein was precipitated using antibodies against NP but was not immunoreactive with anti-globin antibodies indicating that the proper reading frame had not been maintained between the NP and globin sequences.

RESULTS AND DISCUSSION

SECTION I

SYNTHESIS OF NP IN OOCYTES INJECTED WITH THE PLASMID pTKNP

1. Introduction

Recombinant DNA technology has enabled the expression of cloned influenza virus genes in both prokaryotic and eukaryotic hosts (for review see Gething and Sambrook, 1983). The influenza proteins HA (Emtage et al., 1980; Davis et al., 1981; Heiland and Gething, 1981), NS1 (Young et al., 1983) and NP (I. Jones and G. G. Brownlee, unpublished data) have all been expressed in E. coli as has a truncated form of NA (Jones and Brownlee, unpublished data). Expression of influenza genes in eukaryotes has relied upon the use of SV40 vector systems in which the influenza gene replaces either the early or late regions of the SV40 genome. In this way the influenza proteins HA (Gething and Sambrook, 1981; Sveda and Lai, 1981; Hartman et al., 1982), M (Lamb and Lai, 1982) and NP (Lin and Lai, 1983) have been expressed in cultured cells.

This section describes the synthesis and identification of a protein directed by the plasmid pTKNP after injection into Xenopus oocytes. The plasmid pTKNP contains the NP gene from influenza virus A/NT/60/68 under the control of eukaryotic expression elements.

2. Results

When injected into Xenopus oocytes the plasmid pTKNP directed the synthesis of a new protein. This protein was identified as the influenza NP by its migration by SDS-PAGE and its reactivity to both polyclonal and monoclonal (van Wyke et al., 1980) antibodies specific to NP (Figure 46).

To demostrate the specificity of the monoclonal antibodies oocytes were injected with mRNA prepared from CEF cells infected with FP/R virus. Like the NP sythesised from pTKNP the NP synthesised from the mRNA was precipitated by polyclonal anti-NP antibody but exhibited different reactivities to the monoclonal antibodies (Figure 47).

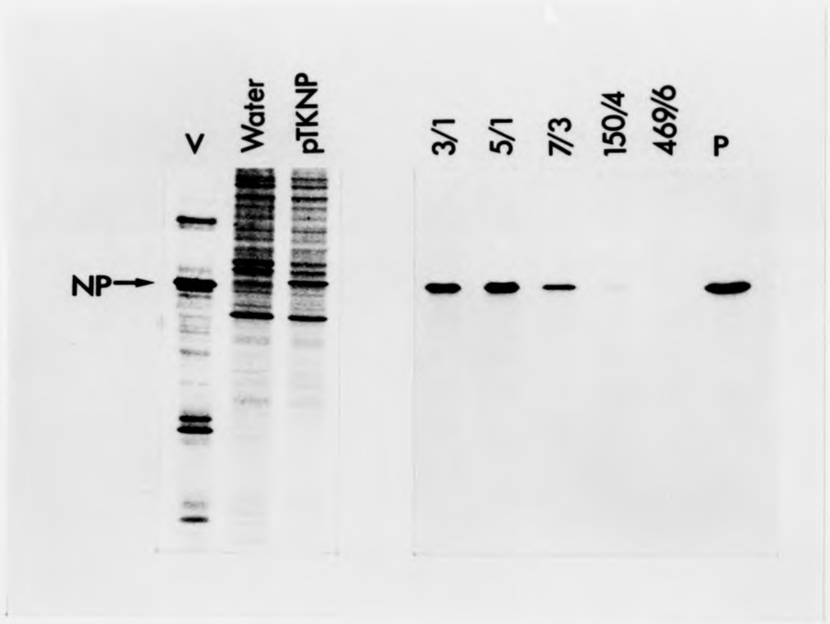


Figure 46 Synthesis of NP following the injection of pTKNP into oocytes

SDS-PAGE analysis of oocytes injected with water or pTKNP. Track V contains ^{35}S-methionine-labelled lysate from CEF cells infected with FP/R virus as marker. The pTKNP-injected oocytes were immunoprecipitated using both polyclonal (P) and monoclonal antibodies. The notation for the monoclonal antibodies is as in van Wyke et al. (1980). The equivalent of one oocyte was loaded per track.

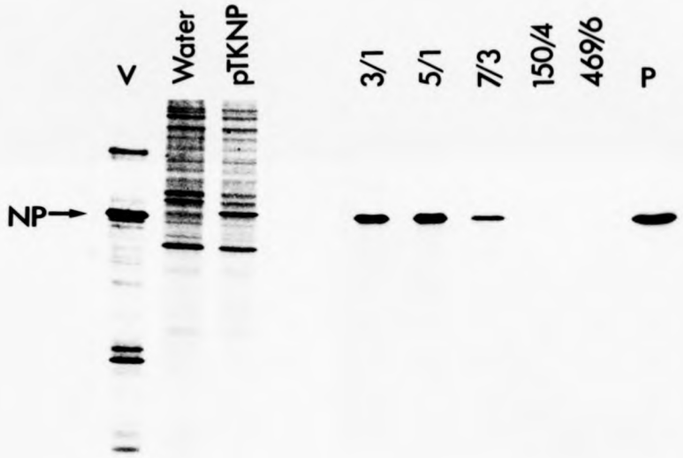


Figure 46 Synthesis of NP following the injection of pTKNP into oocytes

SDS-PAGE analysis of oocytes injected with water or pTKNP. Track V contains ^{35}S-methionine-labelled lysate from CEF cells infected with FP/R virus as marker. The pTKNP-injected oocytes were immunoprecipitated using both polyclonal (P) and monoclonal antibodies. The notation for the monoclonal antibodies is as in van Wyke et al. (1980). The equivalent of one oocyte was loaded per track.

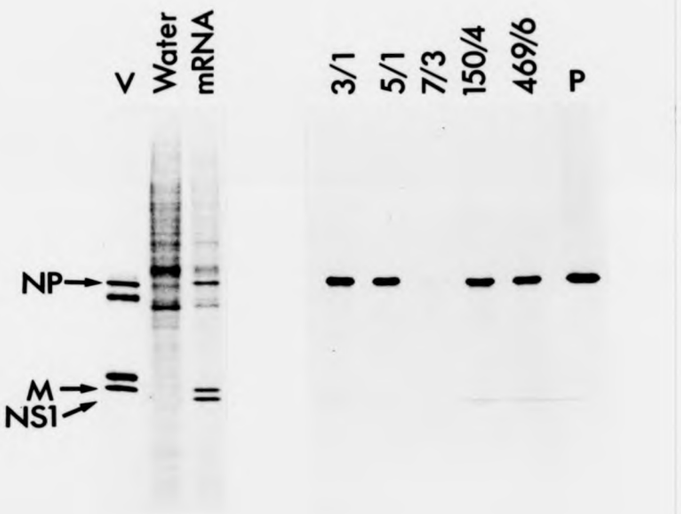


Figure 47 Synthesis of NP following the injection of viral mRNA into oocytes

SDS-PAGE analysis of oocytes injected with water or mRNA prepared from CEF cells infected with FP/R virus. Track V contains purified ^{35}S-methionine FP/R virus as marker. The mRNA-injected oocytes were immunoprecipitated using both polyclonal (P) and monoclonal antibodies. The notation for the monoclonal antibodies is as in van Wyke *et al.* (1980). The equivalent of one oocyte was loaded per track.

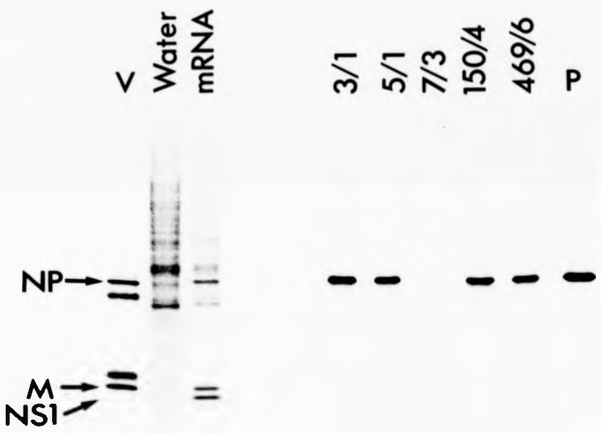


Figure 47 Synthesis of NP following the injection of viral mRNA into oocytes

SDS-PAGE analysis of oocytes injected with water or mRNA prepared from CEF cells infected with FP/R virus. Track V contains purified ^{35}S-methionine FP/R virus as marker. The mRNA-injected oocytes were immunoprecipitated using both polyclonal (P) and monoclonal antibodies. The notation for the monoclonal antibodies is as in van Wyke et al. (1980). The equivalent of one oocyte was loaded per track.

3. Discussion

The protein expressed from pTKNP after injection into oocytes had a MW of 56,000. It was precipitated by polyclonal anti-NP antibody and by the monoclonal antibodies 3/1, 5/1 and 7/3. The NP gene in pTKNP was derived from the influenza strain A/NT/60/68 and the reactivity of the oocyte-synthesised NP with the monoclonal antibodies is the same as that observed for the NPs of influenza viruses isolated at the same time as A/NT/60/68 (van Wyke et al., 1980).

The 56,000 dalton protein synthesised in oocytes injected with mRNA prepared from CEF cells infected with FP/R virus was also precipitated by polyclonal anti-NP antibody but was precipitated by the monoclonal antibodies 3/1, 51, 150/4 and 469/6. This is the same reactivity with the monoclonal antibodies as the NP from influenza A/PR/8/34 (van Wyke et al., 1980) which was isolated at the same time as FP/R (Fowl Plague/Rostock/34).

In conclusion therefore, oocytes injected with pTKNP synthesise a protein of 56,000 daltons which is antigenically identical to the NP of influenza A/NT/60/68.

SECTION II

OPTIMISATION OF PROTEIN PRODUCTION IN OOCYTES INJECTED WITH DNA

1. Introduction

This section describes experiments to determine the amount of DNA which has to be injected into oocytes in order to obtain maximum protein production. It also describes differences in NP production observed when oocytes were injected with DNA prepared by two different methods.

Since the level of protein produced from DNA injected into oocytes isolated from different frogs is variable (Asselbergs *et al.*, 1983; Colman, 1984a) all of the experiments described in this section were performed using the same batch of oocytes.

2. Methods

The CsCl-gradient method of preparing DNA for injection into oocytes is described here, rather than with all the other methods used in the thesis, since this is the only time it was used. The DNA was kindly prepared by Mr. D. Jackson (University of Warwick).

A 500 ml culture of bacteria was grown as described previously and the bacterial pellet was resuspended in 14.5 ml of ice cold resuspension buffer (50 mM Tris, pH 8.0, 25% sucrose and 1.75 mg/ml lysozyme). This mixture was left on ice for 5 min with occasional mixing, made to 75 mM EDTA, pH 8.0 and incubated on ice for a further 5 min. 20 ml of ice cold lysis buffer (50 mM Tris, pH 8.0, 75 mM EDTA, 0.1% Triton X-100) was added and after thorough mixing the tube was centrifuged at 65,000g for 30 min at 4°C. The supernatant was collected, made to 0.2 M NaCl, extracted twice with phenol:chloroform, twice with chloroform and dialysed against 1 mM Tris, pH 8.5, 1 mM EDTA for 3 h with several changes of buffer. The sample was ethanol precipitated and after collection by centrifugation the precipitate was resuspended in 10 ml of 1/10th SSC (15 mM NaCl, 1.5 mM trisodium citrate). Digestion of the RNA was attained by the addition of 60 Kunitz units of RNase A and 300 units of T1 RNase and incubating the sample at 37°C for 1 h with continuous agitation. After being made to 0.3 M NaCl and extracted twice with phenol:chloroform and twice with chloroform, the DNA was collected by ethanol precipitation. The pellet was disolved in 5 ml of 1/10th SSC and loaded onto 25 ml of CsCl at 1.3 mg/ml in 1/10th SSC supplemented with ethidium bromide at 5 mg/ml. Centrifugation was at 240,000g for

16 h and the band corresponding to the closed circular DNA was collected by piercing the tube with a 23 gauge needle. After extracting the ethidium bromide with iso-amyl alcohol the sample was dialysed against 10 mM Tris, pH 8.5, 1 mM EDTA for 3 h with several changes of buffer. The DNA was collected by ethanol precipitation and further purified on a 10-40% sucrose gradient as described previously.

3. Results

Oocytes were injected with various amounts of DNA and the amount of protein produced was determined. The volume injected into each oocyte was kept constant at 25 nl. This is larger than the 10 nl suggested by Wickens and Gurdon (1983), but had no adverse effect on the oocytes as judged by their viability and the structural integrity of the nucleus after injection.

The plasmid pTKNP was diluted to different concentrations in distilled water and injected into oocytes. Injection, radiolabelling, homogenisation and immunoprecipitation with anti-NP antibody were performed as described in 'Methods A'. The immunoprecipitations were designed to precipitate all of the NP in the samples. After analysis by SDS-PAGE (Figure 48) the NP bands were excised from the dried gels and the amount of radioactivity in each band was determined. The relative amount of NP produced in each sample is shown in Figure 49.

The results show that maximum NP production was obtained in oocytes injected with 3.75 ng of pTKNP, that is, about 300 times the total chromosomal DNA content (12 pg) of the (tetraploid) oocyte. When supra-optimal amounts of pTKNP were injected inhibition of NP production was observed. This dose-response relationship is not oocyte specific since oocytes prepared from another frog gave the same results (data not shown), and it is not unique to the plasmid pTKNP since similar results were seen using the plasmid pTKNP Acc 1250 (Figure 50).

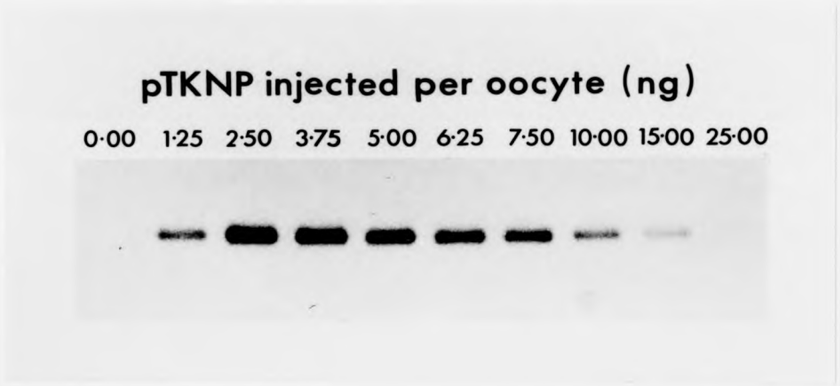


Figure 48 SDS-PAGE analysis of oocytes injected with various amounts of the plasmid pTKNP

DNA was prepared by the alkali-SDS method. The oocytes were injected, radiolabelled, homogenised and immunoprecipitated with the anti-NP antibody as described in 'Methods A'. The equivalent of one oocyte was loaded in each track.

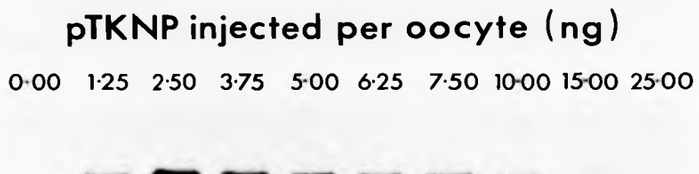


Figure 48 SDS-PAGE analysis of oocytes injected with various amounts of the plasmid pTKNP

DNA was prepared by the alkali-SDS method. The oocytes were injected, radiolabelled, homogenised and immunoprecipitated with the anti-NP antibody as described in 'Methods A'. The equivalent of one oocyte was loaded in each track.

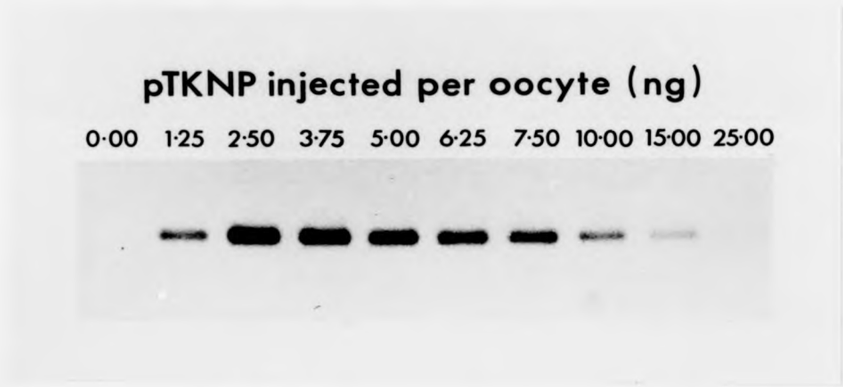


Figure 48 SDS-PAGE analysis of oocytes injected with various amounts of the plasmid pTKNP

DNA was prepared by the alkali-SDS method. The oocytes were injected, radiolabelled, homogenised and immunoprecipitated with the anti-NP antibody as described in 'Methods A'. The equivalent of one oocyte was loaded in each track.

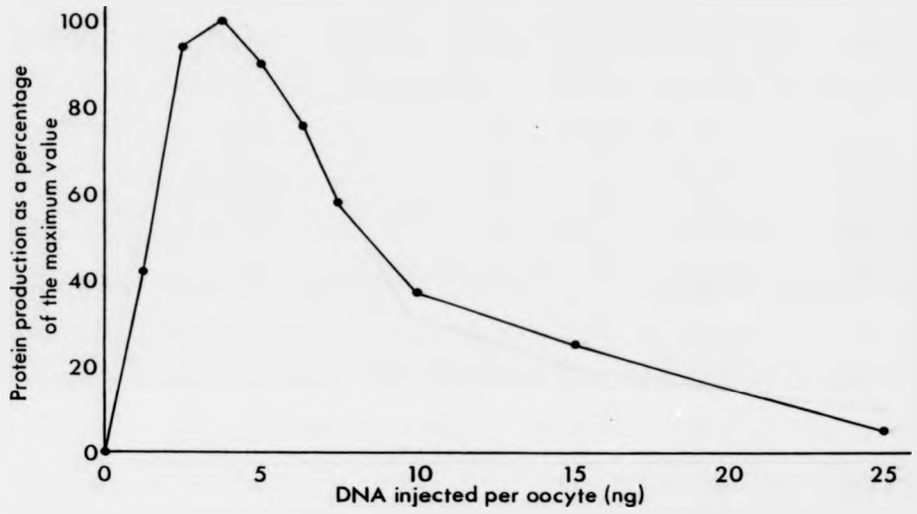


Figure 49 Relationship between the amount of NP produced in oocytes and the amount of pTKNP injected

pTKNP was prepared by the alkali-SDS method. Quantitation of the amount of NP produced is described in 'Methods A'.

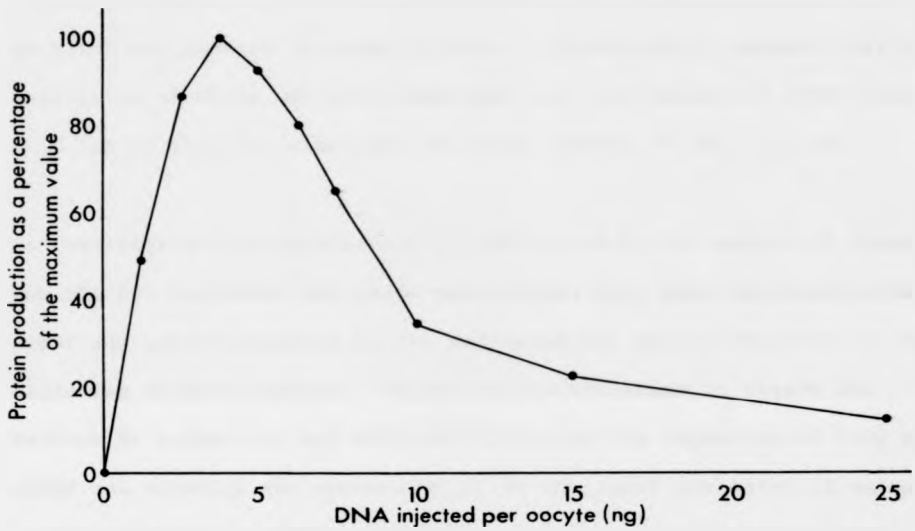


Figure 50 Relationship between the amount of NP Acc 1250 produced in oocytes and the amount of pTKNP Acc 1250 injected

pTKNP Acc 1250 was prepared by the alkali-SDS method.

When the amount of DNA injected into each oocyte was kept constant (at 25 ng/oocyte) by the co-injection of carrier pBR322 DNA (prepared by the alkali-SDS method) (Figure 51) the maximum amount of NP produced was reduced compared to oocytes injected with pTKNP alone. It also seems that the inhibition of NP production observed with supra-optimal amounts of pTKNP was reduced in these oocytes. These results suggest that the production of NP is not only dependent upon the amount of pTKNP injected into the oocytes but also upon the total amount of DNA injected.

To investigate if NP production is influenced by the method of preparing the DNA for injection the above experiments were also performed with pTKNP and pBR322 prepared by the CsCl-gradient method described at the beginning of this section. These results are shown in Figure 52. Maximum NP production was obtained following the injection of 6.25 ng of pTKNP and although the production of NP was again inhibited at supra-optimal amounts of pTKNP, the degree of inhibition was less than with pTKNP prepared by the alkali-SDS method (see Figure 49). When the amount of DNA injected per oocyte was kept constant by the co-injection of carrier pBR322 the production of NP did not significantly change from that observed with the pTKNP alone.

The maximum production of NP from pTKNP prepared by the alkali-SDS method was 76% of the maximum obtained from pTKNP prepared by the CsCl-gradient method.

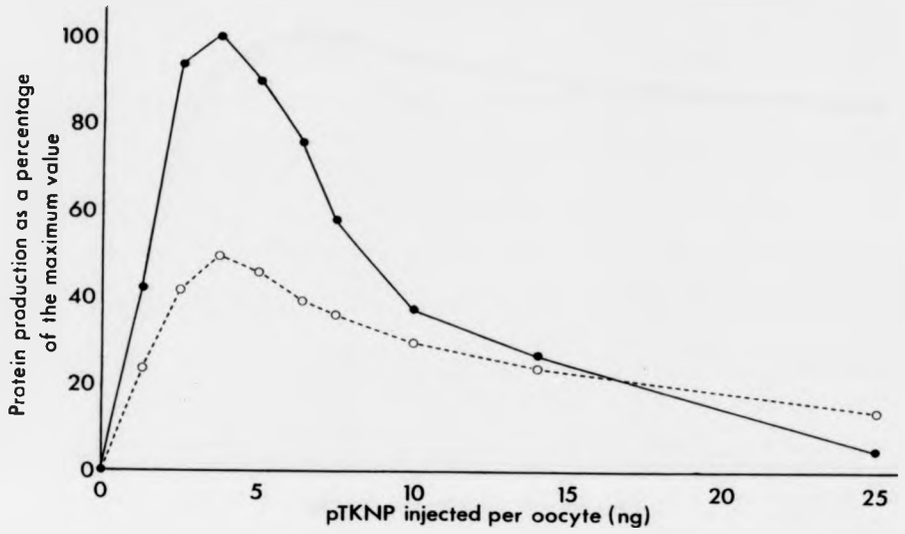


Figure 51 Relationship between the amount of NP produced in oocytes and the amount of DNA injected (alkali-SDS preparation)

The filled symbols represent the assay where different amounts of pTKNP were injected while the open symbols represent the assay where the DNA concentration was kept constant at 25 ng per oocyte using pBR322 DNA as carrier. Both pTKNP and pBR322 were prepared by the alkali-SDS method.

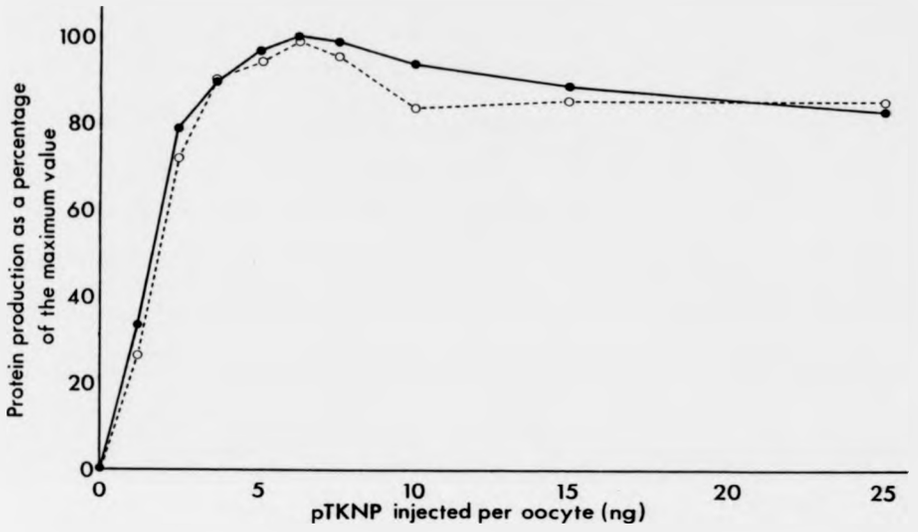


Figure 52 Relationship between the amount of NP produced in oocytes and the amount of DNA injected (CsCl-gradient preparation)

The filled symbols represent the assay where different amounts of pTKNP were injected while the open symbols represent the assay where the DNA concentration was kept constant at 25 ng per oocyte using pBR322 DNA as carrier. Both pTKNP and pBR322 were prepared by the CsCl-gradient method.

4. Discussion

Fully grown Xenopus oocytes contain approximately 12 pg of chromosomal DNA but their high content of RNA polymerase II (Roeder, 1974) is responsible for their ability to rapidly transcribe very large amounts of injected DNA. Indeed there are several reports that maximum transcription from genes normally transcribed by RNA polymerase II is obtained following the injection of between 6 ng (Mertz and Gurdon, 1977) and 10 ng (Gurdon and Melton, 1981; Colman, 1984a) of DNA per oocyte. This is in contrast to the situation with ribosomal DNA (transcribed by RNA polymerase I) where maximum transcription is obtained at 25-50 pg of DNA per oocyte and no transcripts are detectable in oocytes injected with 10 ng of DNA (Moss, 1982). The reasons for these differences are not clearly understood but the necessity of injecting such low amounts of rDNA have been recently disputed (R. Roeder, personal communication to A. Colman). Although not proven, the fact that transcription from all my constructs is under the control of the promotor from the thymidine kinase gene, indicates RNA polymerase II is the enzyme resposible (McKnight and Gavis, 1980) (see 'Results', Section II). It might therefore have been predicted that maximum transcription would occur following the injection of between 6 and 10 ng of pTKNP per oocyte. Indeed the results show that maximum NP production occured in oocytes injected with either 3.75 ng or 6.25 ng of plasmid depending upon the method of DNA preparation. However, since the level of transcription was not studied it is not possible to conclude whether or not maximum transcription is coincident with the maximum production of protein.

When pTKNP, prepared by the CsCl-gradient method, was injected into oocytes at supra-optimal amounts there was a slight inhibition of NP production (Figure 52). This inhibition was not altered by the co-injection of carrier pBR322 DNA suggesting that it was due to a gene dosage effect, possibly resulting from competition for a common factor such as RNA polymerase II. The same effect has also been observed following the injection of supra-optimal amounts of adenovirus DNA (either pronase-treated whole adenovirus DNA or plasmid containing the E2A gene) into Xenopus oocytes (Asselbergs et al., 1983). Again the inhibition was not affected by the co-injection of carrier pBR322 DNA. In this case the plasmid DNA was prepared by the rapid boiling procedure of Holmes and Quigley (1981), and the supercoiled form was further purified by banding in CsCl.

As well as being dependant upon the amount of pTKNP injected into the oocytes the production of NP also appears to depend upon the method used to prepare the DNA. When pTKNP was prepared by the alkali-SDS method the maximum production of NP was reduced compared to that from pTKNP prepared by the CsCl-gradient method and was further reduced by the co-injection of carrier pBR322 DNA also prepared by the alkali-SDS method. This indicates that NP production in oocytes injected with DNA prepared by the alkali-SDS method is not only dependant upon the amount of pTKNP injected but also upon the total amount of DNA injected.

Why the dependence of NP production on the total amount of DNA injected should be more noticeable with DNA prepared by the alkali-SDS method is not known. One possible explanation is that the DNA prepared by the

CsCl-gradient method is more free of contaminating products which inhibit NP production. Whether these inhibitors act at the level of transcription or at the level of translation is not known. It is also not known if these inhibitors are of bacterial origin or are introduced during the isolation of the DNA.

NP was produced in all pTKNP-injected oocytes, but since the lowest amount of DNA injected was 1.25 ng it is not possible to conclude whether or not a lower threshold value exists as was found with adenovirus DNA (Asselbergs et al., 1983). Injection of less than 0.5 ng of adenovirus DNA per oocyte gave no detectable protein production. It is not known however, whether this threshold effect is a consequence of measuring gene expression at the protein level or whether it is specific for the adenovirus DNA since no threshold effect was observed in studies on the transcription of SV40 DNA with RNA polymerase II (Mertz and Gurdon, 1977).

Under optimal conditions the NP contained 7.8% of the total radioactivity incorporated into pTKNP-injected oocytes. This compares very favourably with the 1-2% obtained for the E2A 75K protein in oocytes injected with adenovirus DNA (Asselbergs et al., 1983), the 0.25-1% obtained for VP1 after injection of SV40 DNA (Rungger and Turler, 1978) and the 0.01% obtained for ovalbumin after injection of a cloned chicken ovalbumin gene (Wickens et al., 1980).

For the routine production of DNA for injection it was felt that the speed of the alkali-SDS method outweighed the advantages of the more

involved CsCl-gradient method. The oocytes were injected with between 3 and 4 ng of DNA, the optimum for protein production.

SECTION III

PRODUCTION OF NP FROM A VARIETY OF VECTOR/PROMOTOR COMBINATIONS

1. Introduction

Whereas all of the other sections in this thesis describe results obtained using the expression vector pTK_2 with the protein coding region downstream of the thymidine kinase promotor, this section describes the production of NP in a variety of vector/promotor combinations.

2. Plasmid Construction

a) pTKNP

This is described in detail in 'Methods B' and is again illustrated in Figure 53.

b) pTKNP Minus

The construction of pTKNP Minus was identical to that of pTKNP except that clones were selected in which the NP coding region was inserted into pTK_2 in the opposite polarity to the TK promotor (Figure 53).

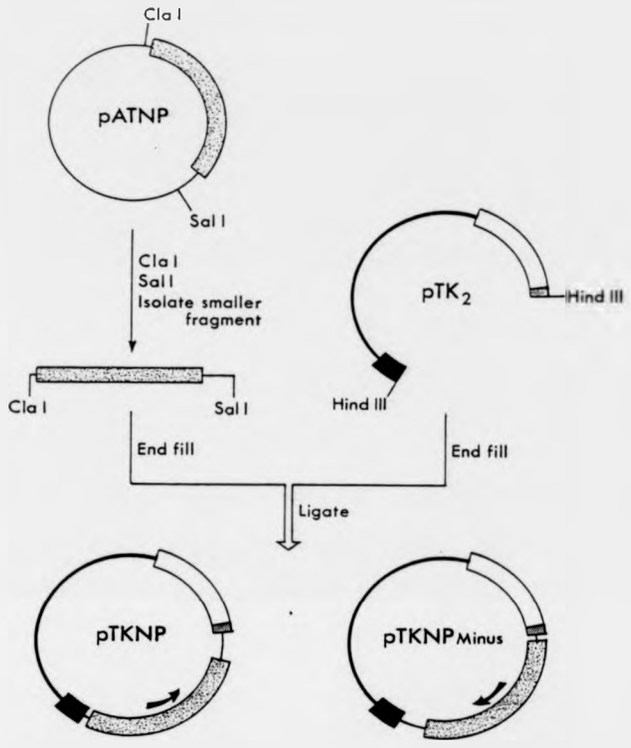


Figure 53 Construction of the plasmids pTKNP and pTKNP Minus

See text for details of the construction. The key for the various elements is as in Figure 10 (see 'Methods B'), and is repeated below for convenience. Sequences from pAT/Pvu II/8 (thin line); pBR322 region (thick line); TK promotor region (solid box); NP gene (stipled box); Stop region (hatched box) and SV40 region (open box). The arrows indicate the 5'→3' direction of the NP coding strand.

c) pNP

The vector pTK_2 was restricted with Pvu II and Hind III and the larger fragment was isolated by gel electrophoresis. After end filling the Hind III site, this fragment was ligated to the same fragment containing the NP coding region that was used in the construction of pTKNP. Clones were selected in which the NP coding sequence was in the same polarity as the TK promotor (Figure 54).

d) pNP Minus

The construction of pNP Minus was identical to that of pNP except that clones were selected in which the NP coding sequence was in the opposite polarity to the TK promotor (Figure 54).

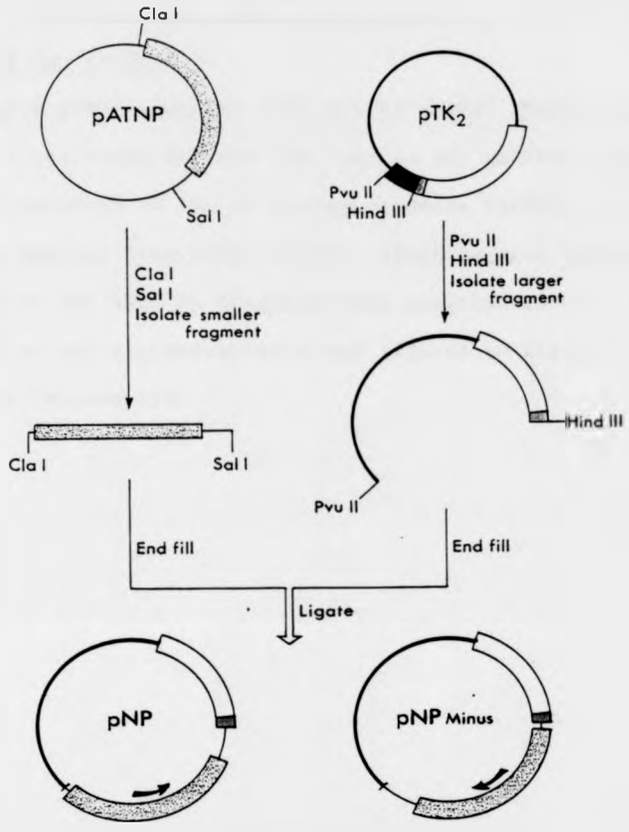


Figure 54 Construction of the plasmids pNP and pNP Minus

See text for details of the construction and Figure 53 for the key to the various elements. The arrows indicate the 5'→3' direction of the NP coding strand.

e) pTKNP Bam 1839/2376

The plasmid pTKNP contains four Bam Hl sites: two in the NP coding region at positions 577 and 793, one in the pAT/Pvu II/8 sequence at the carboxyl terminus of the NP coding sequence (1839) and one in the sequence derived from SV40 (2376). After partial digestion of pTKNP with Bam Hl the 4886 bp fragment from position 2376 to position 1839 was isolated by gel electrophoresis and ligated to itself to give pTKNP Bam 1839/2376 (Figure 55).

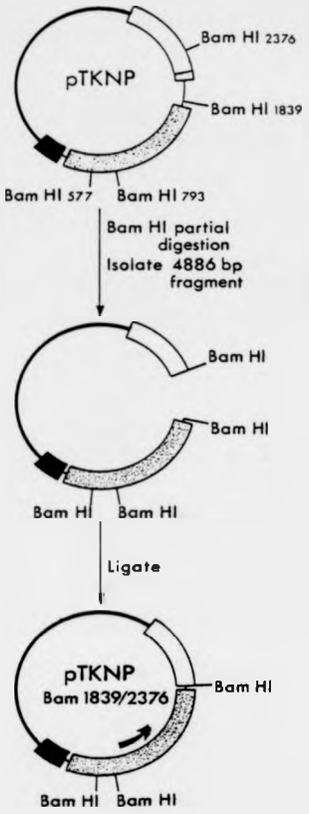


Figure 55 Construction of the plasmid pTKNP Bam 1839/2376

See text for details of the construction. The key for the various elements is as Figure 53. The arrow indicates the 5'→3' direction of the NP coding strand.

f) pTKNP HB 2118/2376 Minus

The vector pTK_2 was restricted with Bam Hl and Hind III and the larger fragment was isolated by gel electrophoresis. After end filling the restriction sites, this fragment was ligated to the same fragment containing the NP coding region that was used in the construction of pTKNP. Clones were selected in which the NP coding region was in the opposite polarity to the TK promotor (Figure 56).

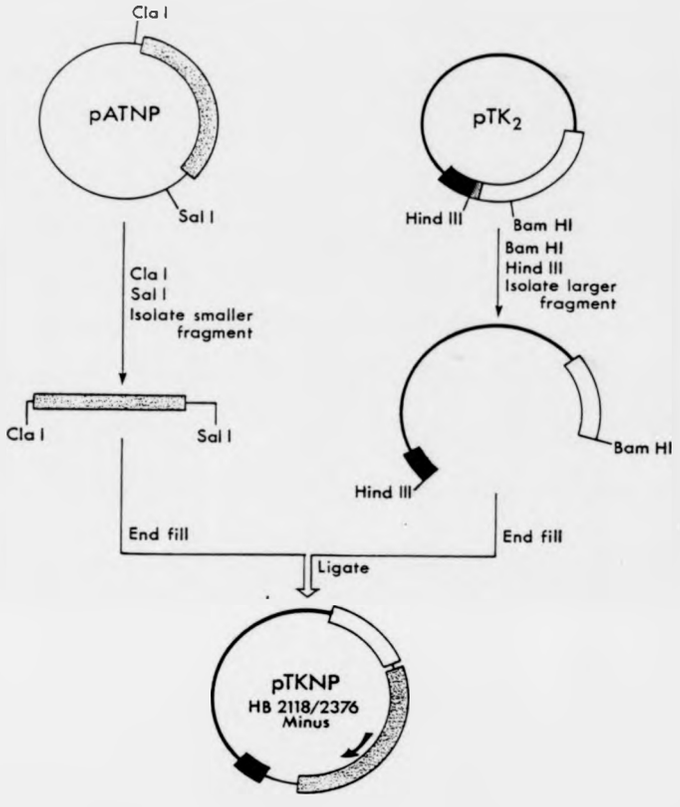


Figure 56 Construction of the plasmid pTKNP HB 2118/2376 Minus

See text for details of the construction. The key for the various elements is as Figure 53. The arrow indicates the 5'→3' direction of the NP coding strand.

3. Results

Each of the plasmids described above were injected into oocytes at 3.75 ng of DNA per oocyte. All of the oocytes were obtained from the same frog. The oocytes were radiolabelled, homogenised and immunoprecipitated with anti-NP antibodies as described in 'Methods A'. The immunoprecipitated samples were analysed by SDS-PAGE (Figure 57) and the relative amount of NP protein produced in each sample was calculated by determining the amount of radioactivity in each NP band. These results are shown in Table 11.

NP was produced from all of the plasmids except pTKNP Bam 1839/2376 and pTKNP HB 2118/2376 Minus.

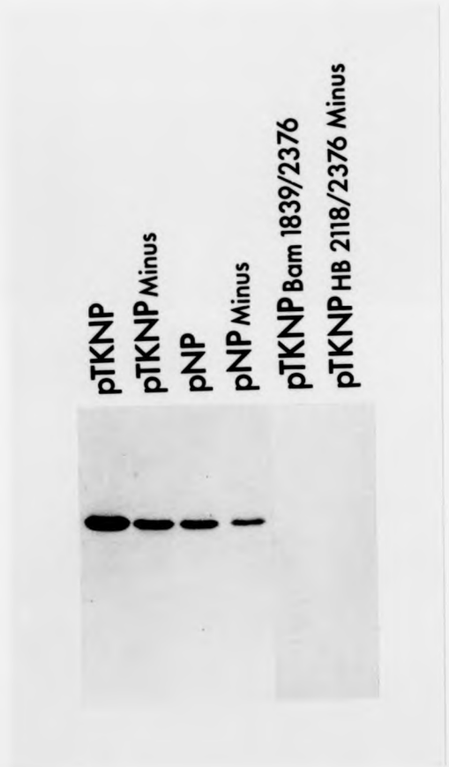


Figure 57 SDS-PAGE analysis of the NP produced by oocytes injected with plasmids containing different constructions of the vector pTK_2

See text for details of the injection and analysis of the oocytes. After immunoprecipitation the equivalent of one oocyte was loaded in each track.

Figure 57 SDS-PAGE analysis of the NP produced by oocytes injected with plasmids containing different constructions of the vector pTK_2

See text for details of the injection and analysis of the oocytes. After immunoprecipitation the equivalent of one oocyte was loaded in each track.

Table 11 Relative production of NP in oocytes injected with plasmids containing different constructions of the vector pTK_2

Plasmid	Percentage relative production of NP
pTKNP	100
pTKNP Minus	10.6
pNP	11.8
pNP Minus	5.8
pTKNP Bam 1839/2376	0
pTKNP HB 2118/2376 Minus	0

4. Discussion

Interpretation of the results is necessarily inconclusive since no attempt was made to analyse the transcripts synthesised from the various constructs. Other workers however have used Xenopus oocytes to study the transcription of both the thymidine kinase gene (McKnight and Gavis, 1980; McKnight et al., 1981) and plasmids very similar to those described above (Krieg et al., 1984), and using these results it is possible to suggest reasons for my findings at the protein level.

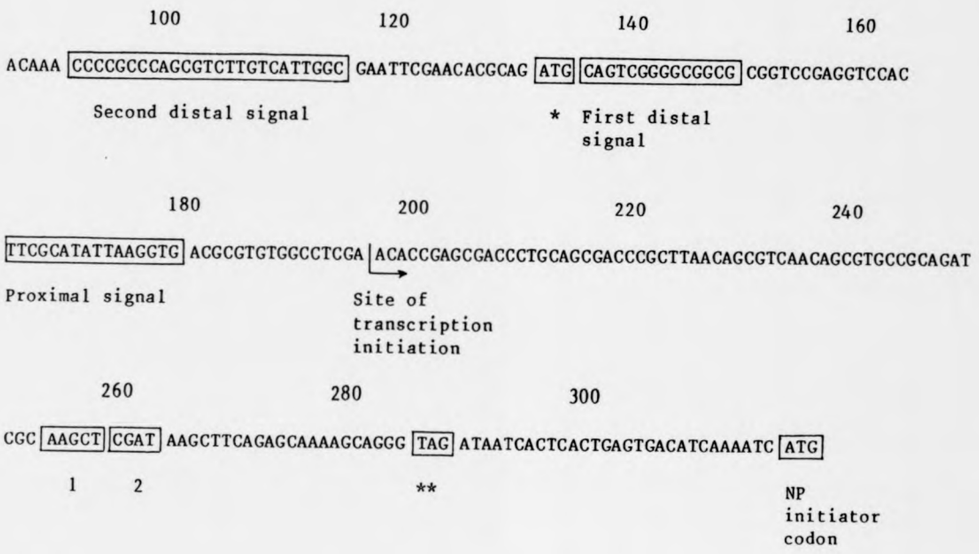
Earlier in the thesis I suggested that the transcripts from pTKNP were produced by RNA polymerase II after initiation at the TK promotor. This promotor is accurately and efficiently transcribed in Xenopus oocytes when present either with the complete TK gene (McKnight and Gavis, 1980; McKnight et al., 1981) or as part of a plasmid containing the eukaryotic expression vector pTK_2 (Krieg et al., 1984) (see Figure 58). The only difference between the plasmid used in the study by Krieg and co-workers, pTKOV+, and pTKNP was the replacement of the chicken ovalbumin coding sequence with that of the influenza NP.

The use of in vitro mutagenesis has defined three spatially distinct regions within the TK promotor that control transcription (McKnight et al., 1981; McKnight, 1982; McKnight and Kingsbury, 1982). These are called the proximal signal, the first distal signal and the second distal signal (see Figure 58). The DNA sequence of the proximal signal harbours the TATA homology for which there is ample evidence for a fundamental role in transcription initiation (for reviews see Corden et

Figure 58 Sequence around the major transcription initiation site of pTKNP and pTKNP Bam 1839/2376

The numbers above the nucleotides indicate their positions in the plasmid pTKNP. The site of transcription initiation is indicated as are the proximal signal, the first distal signal and the second distal signal (McKnight, 1982). The translation initiation codon upstream of the NP initiator is shown. Translation from this initiator is responsible for the production of the extended ovalbumin by pTKOV+ and is presumed to produce a 51 amino acid peptide in oocytes injected with pTKNP. The translation terminator for this peptide is also indicated.

Figure 58



* = initiator codon for the peptide of 51 amino acids.
** = terminator codon for the peptide of 51 amino acids.
1 = end-filled Hind III site
2 = end-filled Cla I site

al., 1980; Breathnach and Chambon, 1981). In contrast, the two distal transcriptional signals do not contain strongly conserved sequences and are believed to represent elements that control the efficiency of transcription (McKnight and Kingsbury, 1982).

When the plasmid pTKOV+ was injected into oocytes it not only directed the synthesis of ovalbumin but also of a second, larger protein which was found to be an ovalbumin polypeptide extended by 51 amino acids at the amino terminus (Krieg et al., 1984). This second protein was the result of translational initiation at an upstream in phase AUG in transcripts arising from promotor(s) upstream of the authentic TK promotor sequence (see below). The position of this ATG in the TK region is shown in Figure 58. Although there are no translation termination codons in the sequence between this ATG and the normal ovalbumin initiator in the plasmid pTKOV+ there is one in the corresponding intervening sequence in pTKNP. Translational initiation at this upstream initiator in pTKNP would produce a peptide containing 51 amino acids which would be undetected in the analysis.

During the analysis of pTKOV+ several transcripts were detected which were of higher MW than the transcript initiated at the authentic TK promotor sequence (Krieg et al., 1984). These large transcripts have not been mapped at the nucleotide level, but at least one is presumably responsible for the production of the extended ovalbumin polypeptide discussed above. It is possible that some of these large transcripts are initiated in the pBR322 region of the plasmid (L. Tabe, personal communication). Transcription from these sites could explain the low

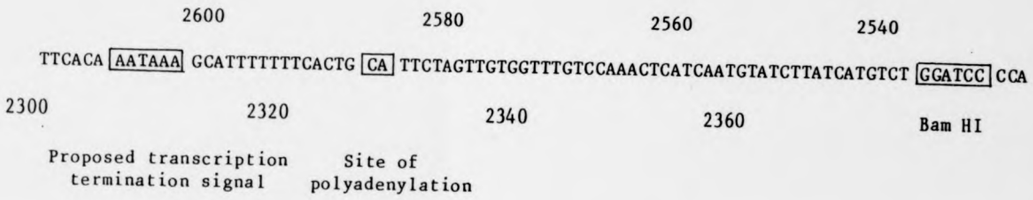
but significant production of NP in oocytes injected with the plasmid pNP in which the TK promotor region is absent. The much greater number of transcripts initiated at the authentic TK promotor compared to those initiated upstream of this region (Krieg et al., 1984) is likely to explain the greater production of NP from pTKNP compared to that from pNP.

The lack of NP production from the plasmid pTKNP Bam 1839/2376 is presumably not due to a lack of transcriptional initiation since the sequences upstream of the NP coding sequence are the same in this plasmid as in the plasmid pTKNP. It seems therefore that the removal of a region downstream of the NP coding sequence results in the loss of NP production. The apparent reason for this becomes evident when the termination of transcription in pTKNP is considered. Although transcription termination has not been analysed either here or in Krieg et al. (1984) it is presumably controlled by the SV40 sequence in the vector pTK_2. Termination and polyadenylation would therefore be identical to that of the early transcripts of SV40 (see Reddy et al., 1979) with polyadenylation occuring at nucleotides 2327/2328 in pTKNP which corresponds to nucleotides 2586/2587 in the SV40 genome (Figure 59). Just upstream of this position is the sequence AATAAA which is common to many polyadenylated eukaryotic mRNAs and may direct the enzymes which cleave the primary transcript and/or which add the poly(A) tract to the new 3' end (see Tooze, 1980). These sequences are absent in pTKNP Bam 1839/2376 (see Figure 59) and their absence may result in the production of transcripts which are either unstable or cannot be translated. The lack of translation could be due to incorrect

Figure 59 Sequence around the transcription termination site of pTKNP and pNP

The numbers below the nucleotides indicate their positions in the plasmid pTKNP while those above the nucleotides indicate their positions in the SV40 genome using the SV numbering system described in Tooze (1980). The site of polyadenylation is indicated (Reddy et al., 1979), as is the sequence proposed to be important in determining the site of termination and/or the site of polyadenylation. The Bam Hl restriction site (position 2376) is indicated for ease of comparison with the other figures.

Figure 59



processing of the transcripts and their subsequent lack of transport into the cytoplasm.

Although pTKNP Bam 1839/2376 contains the complete NP gene including the non-translated flanking sequences which control the initiation and termination of transcription, these signals are presumably inactive in oocytes. During infection the influenza genome remains as RNA and the signals for the initiation and termination of transcription are interpreted by a viral-encoded RNA-dependant RNA polymerase (Schubert et al., 1980; Krug et al., 1981; Robertson et al., 1981). When converted to DNA, as in pTKNP, these signals are different to those that control initiation and termination by the cell's DNA-dependant RNA polymerases.

The plasmid pTKNP Minus contains the same elements as pTKNP but the TK promotor and the NP coding sequence are of opposing polarities. Therefore, it was rather surprising that this plasmid directed the synthesis of NP. A similar result, however, was found using the plasmid pTKOV- (Krieg et al., 1984). This plasmid bears the same relationship to pTKOV+ as pTKNP Minus does to pTKNP, that is, the protein coding region is of the opposite polarity to the TK promotor. To account for the production of the proteins from pTKOV- and pTKNP Minus transcripts of the opposite polarity to the TK transcripts must be synthesised. Indeed the analysis of transcripts from pTKOV- revealed the presence of a "promotor" region in the SV40 sequence (Krieg et al., 1984). The initiation site of these transcripts is shown in Figure 60 which also shows the presence of various promotor-like sequences upstream of this site. Since the SV40 sequence remains intact in both pTKNP Minus and

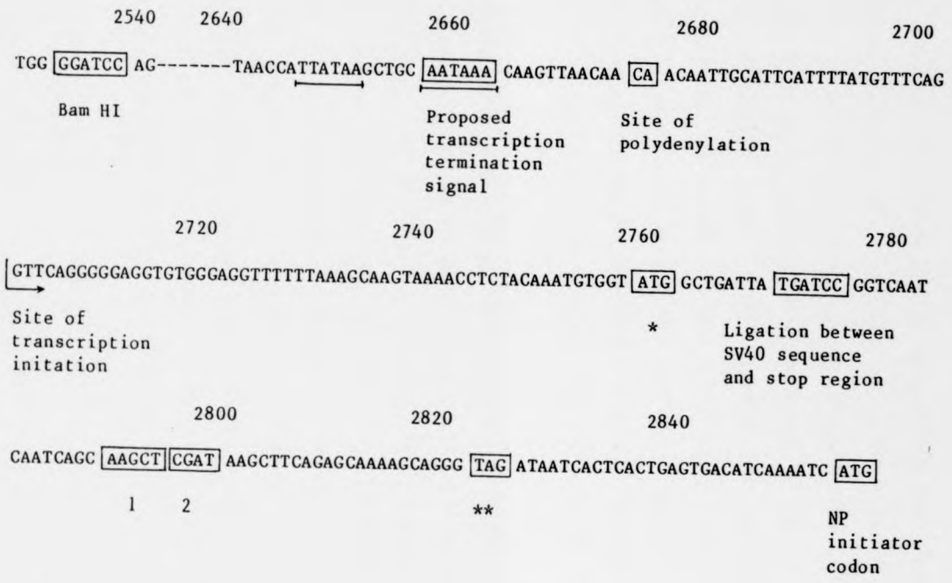
Figure 60 Sequence around the SV40 promotor region active in pTKNP Minus and pNP Minus

The numbers above the nucleotides indicate their positions in the SV40 genome using the SV numbering system described in Tooze (1980). The site of transcription initiation is indicated and the sequences underlined are those which bear a striking resemblance to sequences important in initiating transcription. The translation initiation codon upstream of the NP initiator is shown. Translation from this initiator is rsponsible for the production of the extented ovalbumin by pTKOV- (Krieg et al., 1984), and is presumed to produce a 21 amino acid peptide in oocytes injected with pTKNP Minus and pNP Minus.

Transcripts produced from this promotor are presumed to be terminated by the signals responsible for the termination of the SV40 late transcripts. The sequence proposed to be important in determining the site of transcription termination and/or the site of polyadenylation is indicated as is the site of polyadenylation.

In the plasmid pTKNP HB 2118/2376 Minus the region between the Bam H1 site and the end-filled Cla 1 site is absent.

FIGURE 60



* = initiator codon for the peptide of 21 amino acids
** = terminator codon for the peptide of 21 amino acids
1 = end-filled Hind III site
2 = end-filled Cla I site

pNP Minus it is presumed that the production of NP by these plasmids is a consequence of transcription from this promotor.

It is not known if the use of this SV40 "promotor" reflects a special characteristic of amphibian oocyte nuclei or amphibian cell nuclei in general, but it does not appear to function in cultured mammalian cells (Krieg et al., 1984).

When the plasmid pTKOV- was injected into oocytes it not only directed the synthesis of ovalbumin but also of a second, more abundant, larger protein which was found to be an ovalbumin polypeptide extended by 21 amino acids at the amino terminus (Krieg et al., 1984). This second protein was the result of translational initiation at an upstream in phase AUG in the same transcript that directs the synthesis of ovalbumin. When translation is initiated at two AUG codons in a single transcript two factors govern which initiator is used preferentially. The first of these factors is the position effect in which the 5'-proximal AUG is usually used to initiate translation. This holds true for 95% of eukaryotic mRNAs (Kozak, 1984a). The second factor was revealed by a survey of eukaryotic mRNA sequences (Kozak, 1983) which found that functional initiation codons occur in a restricted sequence context with 5'-ANNAUGG-3' (wild-type ovalbumin) being favoured over 5'-GNNAUGG-3' (extended ovalbumin). The fact that the extended ovalbumin was produced in greater amounts than the wild type ovalbumin suggests that translation is preferentially initiated at the 5'-proximal AUG even though it is in a less favoured sequence context. This dominance of position effect over sequence context has also been reported in

experiments involving the mutagenesis of the 5' non-coding sequence of preproinsulin (Kozak, 1984b). The effects of position and sequence context on translation initiation at a given AUG is discussed more fully in a later section of the thesis.

No extended NP polypeptide was produced from either pTKNP Minus or pNP Minus. Translation initiation presumably still occurs at the 5'-proximal AUG, but as shown in Figure 60 there is a translation termination codon in the non-coding sequence upstream of the NP initiator. The resulting peptide of 21 amino acids would not be detected during the analysis.

NP production from pTKNP is approximately 10-fold greater than that from pTKNP Minus. This is probably a reflection of both the greater level of transcription from the TK promotor (Krieg et al., 1984) and also the fact that in the transcripts produced from the SV40 "promotor" translation inititaion probably occurs preferentially at the AUG codon upstream of the NP initiatior.

As discussed above, the transcripts produced from the SV40 "promotor" are unlikely to be terminated and polyadenylated by signals in the region containing the NP coding sequence. It is more likely that the transcripts are terminated and polyadenylated by signals in the region containing the SV40 sequence. As well as containing the signals to terminate and polyadenylate the SV40 early transcripts this sequence also contains the same signals for the SV40 late transcripts (Reddy et al., 1979) (see Figure 60). As can be seen from Figure 60 this would

mean that the transcripts are virtually complete copies of the plasmid.

The plasmid pTKNP HB 2118/2376 Minus did not direct the synthesis of NP. Presumably this is due to the removal of the SV40 "promotor" (Figure 60).

Conclusion

The results presented in this section are consistent with the existence of at least three promotors in the expression vector pTK_2; the authentic TK promotor which promotes transcription in a anti-clockwise direction (promotor 1 in Figure 61); a promotor in the SV40 sequence which promotes transcription in a clockwise direction (promotor 2), and a promotor which lies upstream of the TK promotor region and promotes transcription in an anticlockwise direction (promotor 3). There also seems to be at least one promotor in the TK region upstream of the authentic TK promotor (promotor 4). The clockwise and anti-clockwise directions refer to pTK_2 as drawn in Figure 61.

There appears to be two regions which control transcriptional termination and polyadenylation. These are both in the SV40 sequence but operate in different polarities. These regions are responsible for controlling the termination and polyadenylation of early and late transcripts of SV40.

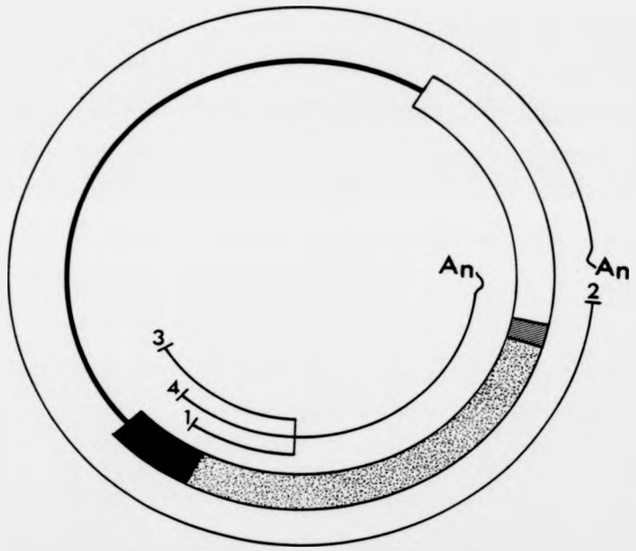


Figure 61 Sites of transcription initiation and termination in the vector pTK_2

To help interpretation an unspecified insert (stipled box) is shown in the vector. The key for the other elements is the same as that used throughout the thesis, that is: TK promotor region (solid box); stop region (hatched box); SV40 region (open box) and the pBR322 region (thick line). The transcripts are indicted by the thin lines. The numbers are used to indicate the sites of transcription initiation:

1 = Transcripts produced from the authentic TK promotor.

2 = Transcripts produced from the SV40 promotor.

3 and 4 = Transcripts produced from promotors upstream of the authentic TK promotor. These transcripts have not been studied in detail but there is evidence that at least some are initiated upstream of the TK promotor region.

SECTION IV

THE ACCUMULATION OF NP IN THE NUCLEI OF *XENOPUS* OOCYTES

1. Introduction

NP was first detected in the nuclei of influenza-infected cells by immunofluorescence staining (Watson and Coons, 1954; Liu, 1955; Breitenfeld and Schäffer, 1957). The analysis of radiolabelled proteins following the fractionation of infected cells revealed that after cytoplasmic synthesis the majority of the NP rapidly migrates to the nucleus where it associates with the nucleoplasm (Dimmock, 1969; Taylor et al., 1969, 1970; Lazarowitz et al., 1971; Krug and Etkind, 1973; Hay and Skehel, 1975; Briedis et al., 1981).

This section describes the accumulation of NP in the nuclei of Xenopus oocytes following its introduction as the protein itself or encoded in mRNA or DNA.

2. Results

a) Injection of NP protein

When CEF cells are infected with FP/R virus and radiolabelled with ^{35}S-methionine for 10 min at 3.5 h p.i. the most heavily radiolabelled protein is NP (Figure 62). Whole lysate prepared from these labelled cells was injected into the cytoplasm of Xenopus oocytes.

At various times after injection the oocytes were enucleated and the isolated nuclear and cytoplasmic fractions were analysed by SDS-PAGE (Figure 63). Calculation of the extent to which NP is concentrated in the nucleus depends upon the relative nuclear and cytoplasmic volumes. As discussed in 'Methods A', although the nucleus occupies 4% of the oocyte volume it has been estimated that it represents 12% of the volume accessible to most macromolecules (Bonner, 1978). This means, for example, that if both compartments contain the same amount of a protein then the concentration in the nucleus is 7.33 fold higher than in the cytoplasm. Figure 64 shows the increase with time of the nuclear to cytoplasmic concentration ratio following the injection of radiolabelled NP into the cytoplasm. The NP became concentrated in the nucleus achieving a maximum ratio of 4.24 after 48 h. Since the total radioactivity incorporated into the NP did not significantly change during the experiment I conclude that little or no degradation of NP occurred in either the cytoplasm or the nucleus.

When the experiment was performed at $4^{o}C$, instead of $20^{o}C$, the NP still entered the nucleus but to a much lesser extent (Figure 64).

Figure 62 Radiolabelled lysate of CEF cells infected with FP/R virus

CEF cells were infected with FP/R virus and radiolabelled with ^{35}S-methionine for 10 min at 3.5 h p.i. The lysate was analysed without dilution and with consecutive two-fold dilutions. Non-infected cell lysate (NI) is included as a control.

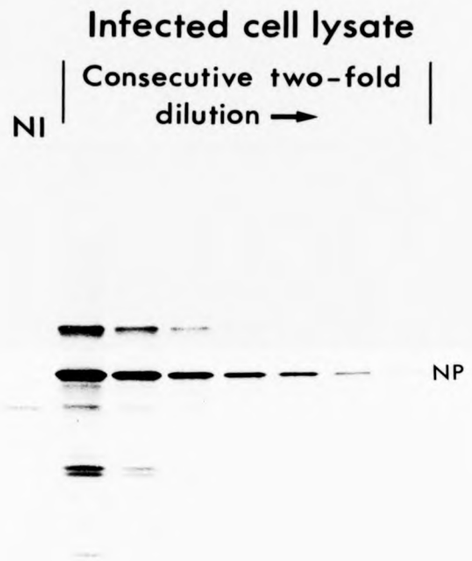


Figure 62 Radiolabelled lysate of CEF cells infected with FP/R virus

CEF cells were infected with FP/R virus and radiolabelled with ^{35}S-methionine for 10 min at 3.5 h p.i. The lysate was analysed without dilution and with consecutive two-fold dilutions. Non-infected cell lysate (NI) is included as a control.

Figure 63 SDS-PAGE analysis of oocytes injected with radiolabelled NP

Radiolabelled lysate from CEF cells infected with FP/R virus was injected into the cytoplasm of Xenopus oocytes. The oocytes were enucleated at various times after injection and the separated nuclear (N) and cytoplasmic (C) fractions analysed by SDS-PAGE. A minimum of ten nuclei and ten cytoplasms were pooled for each time point and the equivalent of one nucleus and one cytoplasm loaded per track.

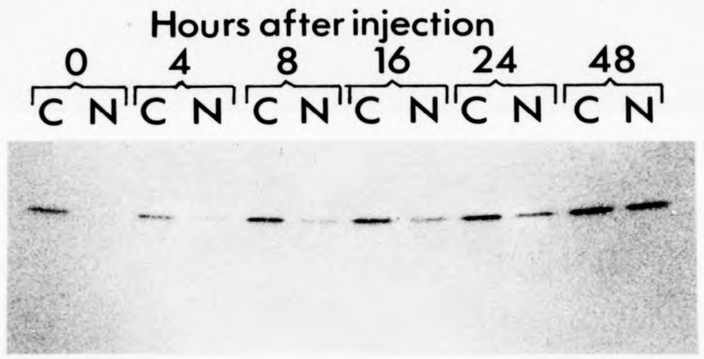


Figure 63 SDS-PAGE analysis of oocytes injected with radiolabelled NP

Radiolabelled lysate from CEF cells infected with FP/R virus was injected into the cytoplasm of Xenopus oocytes. The oocytes were enucleated at various times after injection and the separated nuclear (N) and cytoplasmic (C) fractions analysed by SDS-PAGE. A minimum of ten nuclei and ten cytoplasms were pooled for each time point and the equivalent of one nucleus and one cytoplasm loaded per track.

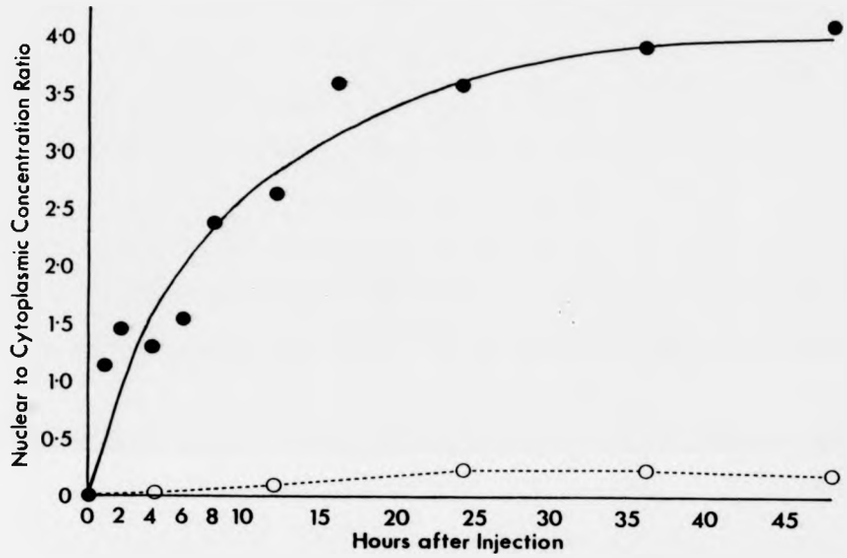


Figure 64 The migration of radiolabelled NP into the nuclei of oocytes

The solid symbols represent the results at 20°C while the open symbols represent those at 4°C.

b) Injection of mRNA

Messenger RNA was prepared from CEF cells infected with FP/R virus and injected into Xenopus oocytes. These oocytes, and control oocytes injected with the same volume of water, were incubated for 20 h to allow the mRNA to become assimilated into polysomes and then labelled for a further 24 h in the presence of ^{35}S-methionine. The oocytes were enucleated and the separated fractions analysed by SDS-PAGE (Figure 65). Three new proteins were synthesised in detectable amounts. From their migration by SDS-PAGE and their precipitation by specific antibodies these were identified as the influenza virus proteins; NP (56 Kd), matrix protein (M) (26 Kd) and the non-structural protein NS1 (25 Kd).

Whereas NS1 is normally concentrated in the nucleoli of infected cells (Dimmock, 1969; Krug and Etkind, 1973; Krug and Soiero, 1975) it remained largely in the oocyte cytoplasm. Both NP and M associated with the oocyte nucleus although only the NP became concentrated there (Table 12). When the nuclear fraction was centrifuged at 12,000g M was pelleted (Figure 65 and Table 12) indicating that it is associated with some nuclear structure. NP was not pelleted under the same conditions.

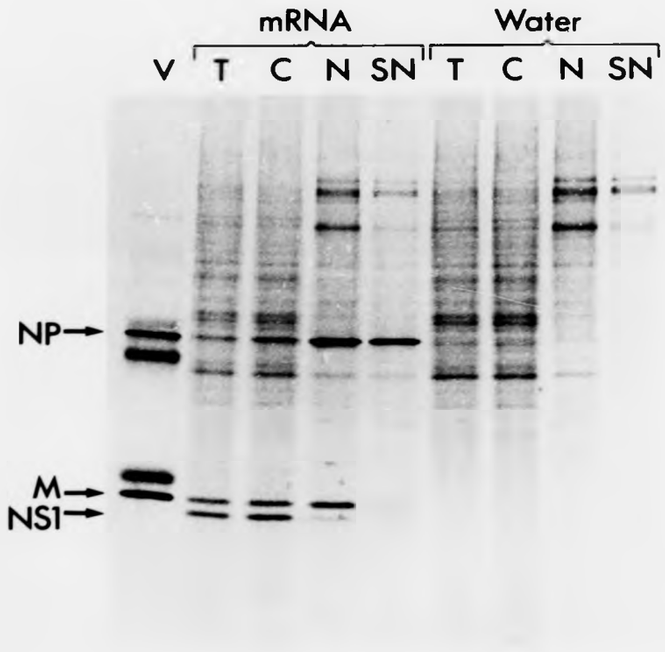


Figure 65 The nuclear association of influenza virus proteins following the injection of viral mRNA into oocytes

Total (T), cytoplasmic (C), nuclear (N) fractions and the supernatant from the centrifugation of the nuclear fraction (SN) of oocytes injected with water or mRNA were analysed by SDS-PAGE. Approximately equal amounts of radioactivity in each track was achieved by loading the equivalent of one total oocyte (T), 1.5 cytoplasms (C) and 10 nuclei (N and SN). Track V contains purified ^{35}S-methionine FP/R virus as marker.

Table 12 The nuclear association of NP, M and NS1 following the injection of viral mRNA into oocytes

Protein	Nuclear to cytoplasmic concentration ratio	'Spun' nuclear to cytoplasmic concentration ratio[a]
NP	3.69	3.55
M	0.95	0.16
NS1	0.29	0.28

[a] Nuclear fraction after centrifugation at 12,000g for 5 min.

c) Injection of DNA

The plasmid pTKNP was injected into Xenopus oocytes. These oocytes, and control oocytes injected with the same volume of water, were incubated for 20 h to allow assembly of chromatin and the accumulation of mRNA before being labelled with ^{35}S-methionine for 6 h. The oocytes were enucleated and the isolated nuclear and cytoplasmic fractions immunoprecipitated using polyclonal anti-NP antibody before being analysed by SDS-PAGE (Figure 66). The NP was 8.27 fold more concentrated in the nucleus than in the cytoplasm (this is the average of six experiments).

To investigate whether or not this eight fold concentration was the maximum attainable by the NP, oocytes injected with pTKNP and radiolabelled as above were chased for 24 h in the absence of ^{35}S-methionine. After enucleation of these chased oocytes the nuclear to cytoplasmic concentration ratio was again calculated (Table 13). Since there was no appreciable change in the ratio it appears that the eight fold concentration in the nucleus represents the NP distribution at equilibrium. The stability of the NP in the oocytes is demonstrated by the fact that the radioactivity incorporated into NP is approximately the same in chased and unchased oocytes (Table 13).

Figure 66 The nuclear accumulation of NP following the injection of pTKNP into oocytes

The isolated nuclear (N) and cytoplasmic (C) fractions of water or pTKNP-injected oocytes were immunoprecipitated using polyclonal antibodies against NP and analysed by SDS-PAGE. One nucleus and one cytoplasm were loaded in each track.

Table 13 Nuclear to cytoplasmic concentration ratios of chased and unchased oocytes injected with pTKNP

	Radioactivity in the NP band		Nuclear to cytoplasmic concentration ratio
	Nucleus	Cytoplasm	
Unchased oocytes	12,462	11,501	7.95
Chased oocytes	13,185	11,506	8.41

The radioactivity values shown represent the counts per minute obtained from the equivalent of one nucleus and one cytoplasm. For convenience the background radioactivity in each track has already been substracted.

d) Injection of cloned NP protein

I demonstrate above that, at equilibrium, the NP synthesised in oocytes from injected pTKNP is over eight fold more concentrated in the nucleus than in the cytoplasm. This ratio is small relative to that observed with other karyophilic proteins in Xenopus oocytes (Gurdon, 1970; Bonner, 1975a; Dingwall et al., 1982). One possible explanation for this low level of accumulation is that at equilibrium the NP in the cytoplasm is prevented from entering the nucleus by binding to a non-nuclear substrate. To investigate this I injected pTKNP into oocytes and prepared radiolabelled NP from both the nucleus (nuc-NP) and the cytoplasm (cyt-NP) of these oocytes. The nuc-NP and cyt-NP were injected separately into the nuclear and cytoplasmic regions of freshly isolated oocytes which were then incubated for 48 h before being enucleated. The isolated nuclear and cytoplasmic fractions were immunoprecipitated with anti-NP antibody and analysed by SDS-PAGE (Figure 67). All of the oocytes in which cyt-NP was injected into the nucleus died within 6 h. In all other cases the radiolabelled NP was found in both cellular compartments but was between six and seven fold more concentrated in the nucleus than in the cytoplasm.

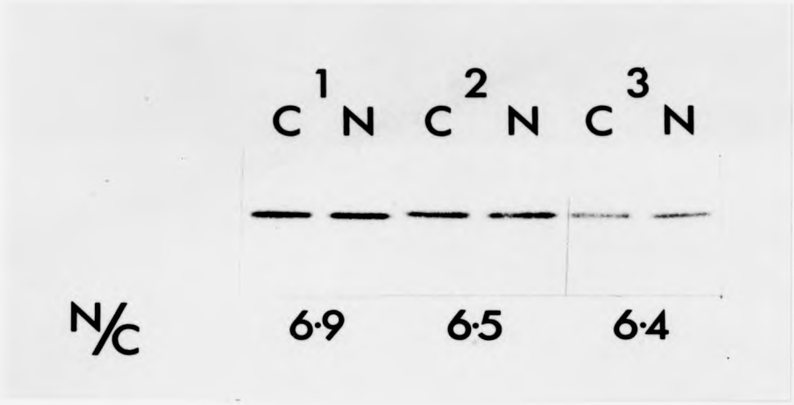


Figure 67 Injection of cloned NP protein into oocytes

Cytoplasmic (C) and nuclear (N) fractions of oocytes injected with cloned NP protein. 1: cyt-NP injected into cytoplasm; 2: nuc-NP injected into cytoplasm; 3: nuc-NP injected into nucleus. Oocytes were analysed individually and representative examples are shown. The nuclear to cytoplasmic concentration ratio (N/C) for each sample was calculated as normal.

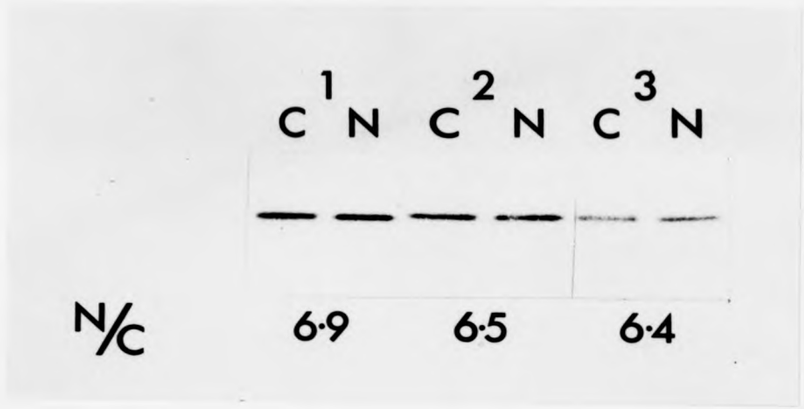


Figure 67 Injection of cloned NP protein into oocytes

Cytoplasmic (C) and nuclear (N) fractions of oocytes injected with cloned NP protein. 1: cyt-NP injected into cytoplasm; 2: nuc-NP injected into cytoplasm; 3: nuc-NP injected into nucleus. Oocytes were analysed individually and representative examples are shown. The nuclear to cytoplasmic concentration ratio (N/C) for each sample was calculated as normal.

e) Injection of pTKNP into tissue culture cells

The plasmid pTKNP was injected into the nuclei of cultured BHK-21 cells. After 8 h the injected cells were fixed and immunofluorescently stained using polyclonal antibody against NP (Figure 68A). As a control, monolayers of BHK-21 cells were infected with influenza virus A/NT/60/68 and fixed and stained 24 h after infection (Figure 68B). The NP produced in the cells injected with pTKNP was predominantly nuclear and the pattern of nuclear fluorescence was the same as that seen in the cells infected with A/NT/60/68. No immunofluorescence was detected in the uninjected cells in Figure 68A.

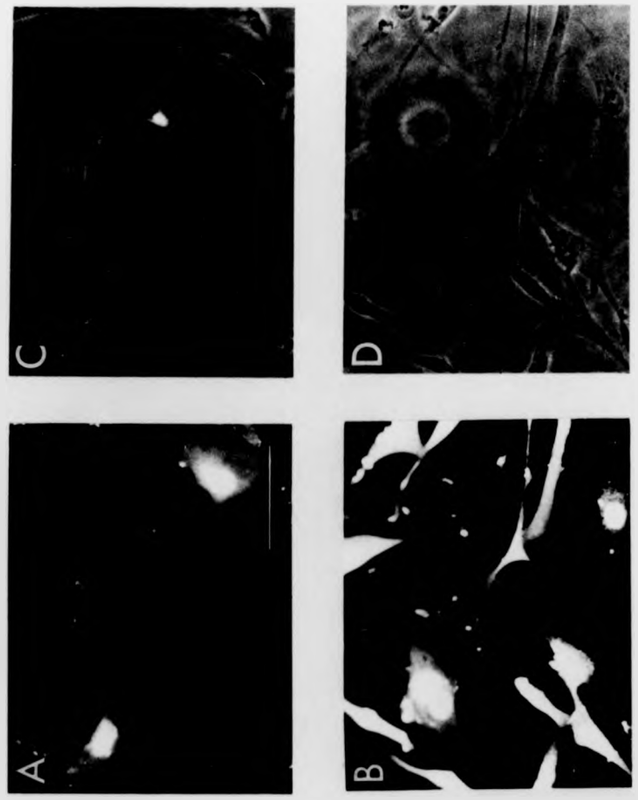


Figure 68 Indirect immunofluorescence of NP in BHK-21 cells

A and C: Injected with pTKNP

B and D: Infected with A/NT/60/68 virus.

The cell monolayers were fixed with acetone and incubated with a polyclonal anti-NP rabbit antibody and subsequently FITC-conjugated goat anti-rabbit serum. The majority of cells in the pTKNP-injected sample are uninjected and serve as internal controls. Figures A and B show the immunofluorescence photographs, while C and D are the corresponding phase contrast images. All photographs are at the same magnification and the bar in Figure A represents 50 µm.

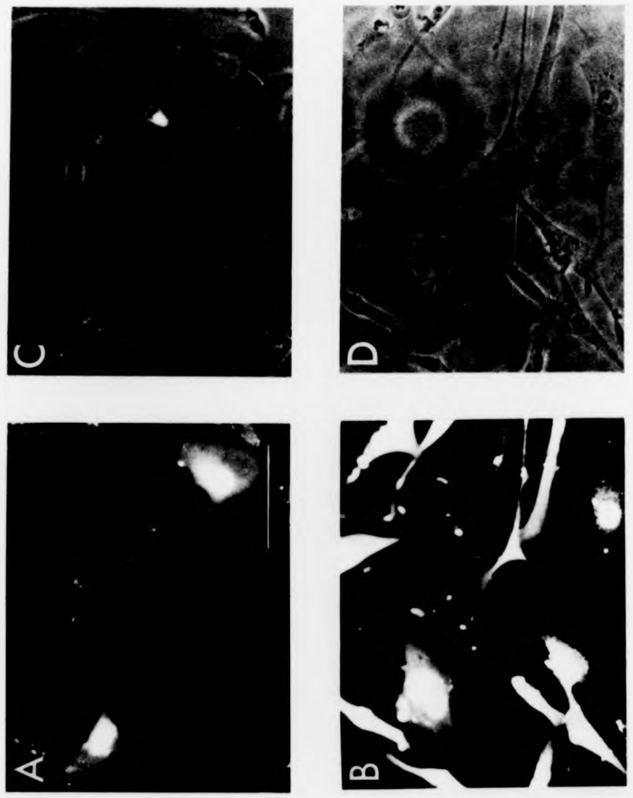


Figure 68 Indirect immunofluorescence of NP in BHK-21 cells

A and C: Injected with pTKNP

B and D: Infected with A/NT/60/68 virus.

The cell monolayers were fixed with acetone and incubated with a polyclonal anti-NP rabbit antibody and subsequently FITC-conjugated goat anti-rabbit serum. The majority of cells in the pTKNP-injected sample are uninjected and serve as internal controls. Figures A and B show the immunofluorescence photographs, while C and D are the corresponding phase contrast images. All photographs are at the same magnification and the bar in Figure A represents 50 µm.

3. Discussion

a) The nuclear association of the influenza virus proteins M and NS1

The matrix protein (M) did not concentrate in the oocyte nucleus but became evenly distributed between this compartment and the cytoplasm. M was removed from the nuclear lysate by centrifugation at 12,000g for 5 min suggesting it is likely to be associated with some relatively large nuclear structure. Since M is associated with cellular membranes during infection (Klenk *et al.*, 1974; Meier-Ewert and Compans, 1974; Hay and Skehel, 1975) and the lipid envelope in the virus particle (Apostolov and Flewett, 1969; Compans and Dimmock, 1969; Gregoriades and Frangione, 1981), and isolated M has a high affinity for lipid *in vitro* (Gregoriades, 1980) this structure could be the nuclear envelope. It is therefore possible that M does not enter the oocyte nucleus but associates with the outer face of the nuclear envelope and that its apparently even distribution between the nucleus and the cytoplasm is fortuitous. In cells infected with influenza virus the extent of nuclear accumulation of M appears to be dependent on both the virus strain and the host cell type being studied (Lazarowitz *et al.*, 1971; Gregoriades, 1973, 1977; Krug and Etkind, 1973; Hay and Skehel, 1975; Flawith and Dimmock, 1979; Mahy *et al.*, 1980; Briedis *et al.*, 1981). Since the mRNA injected into the oocytes was prepared from a virus-cell combination in which M accumulates in the nucleus during infection (Hay and Skehel, 1975; P. J. Rees and N. J. Dimmock, unpublished data) it appears that any property conferred on the M to cause its accumulation in nuclei is either not evident at the level of the mRNA or is different between CEF cells and *Xenopus* oocytes.

The non-structural protein NS1 did not appreciably associate with the oocyte nucleus. This is unlike the situation in cells infected with influenza virus where NS1 migrates to the nucleus (Taylor et al., 1969, 1970; Lazarowitz et al., 1971; Hay and Skehel, 1975). and concentrates in the nucleoli (Dimmock, 1969; Krug and Etkind, 1973; Krug and Soiero, 1975). Since, in the oocytes, the NS1 did not evenly distribute between the nucleus and the cytoplasm it is possible that it is actively prevented from entering the nucleus by being associated with some cytoplasmic structure.

In other regards the oocyte handles influenza proteins normally since in earlier experiments performed in our laboratory the two virus glycoproteins, HA and NA, were enriched in the membrane vesicles of oocytes injected with influenza mRNA (T. Petri, N. J. Dimmock and A. Colman, unpublished data). This agrees with data from cells infected with influenza virus where, like the majority of integral membrane proteins, they are synthesised on the rough endoplasmic reticulum and transported to the plasma membrane via smooth internal membranes (Compans, 1973a; Stanley et al., 1973; Hay, 1974; Klenk et al., 1974).

b) The nuclear association of the influenza virus NP

Regardless of whether the NP was introduced into the oocytes as the protein itself or encoded in RNA or DNA is entered into and accumulated in the nucleus. This accumulation occurs post-translationally with no apparent modification of the protein.

Although the molecular dimensions of NP are unknown its entry into the oocyte nucleus is more rapid than that predicted for a protein of 56,000 daltons (see Bonner, 1978). NP injected into the cytoplasm became evenly distributed between the nucleus and the cytoplasm within about 1 h while injected ovalbumin (44,000 MW) takes 72 h to become evenly distributed, and BSA (67,000 MW) is still not evenly distributed after 72 h (Bonner, 1975a). This rapid entry of NP into oocyte nuclei is consistent with the existence of a mediated transport process as has been suggested for RN1, a 148,000 MW protein of Rana pipiens oocytes (Feldherr et al., 1983).

This is probably an over-simplification of the situation since it is unlikely that proteins exist in cells in a soluble, monomeric state (Fulton, 1982). A detailed mathematical model of protein solutions containing high concentrations of proteins indicates that such solutions are non-ideal (Minton, 1981): the proteins tend to be forced into compact configurations rather than extended ones, and the solutions favour self- and hetero-associations that would be less frequent in dilute solutions. Xenopus oocytes appear to be an extreme example of a non-ideal solution since the cytoplasm, excluding the yolk proteins, is 30-40% protein by weight and it has been estimated that less than 20% of the cytoplasmic protein, excluding yolk proteins, is able to diffuse freely throughout the oocyte (Paine, 1982). The NP has the added complication of being associated with viral RNA to form ribonucleoprotein (RNP) complexes in both the virus particle (Duesberg, 1969; Pons et al., 1969; Compans, et al., 1972; Rees and Dimmock, 1981a) and infected cells (Pons, 1971, 1975; Caliguiri and Gerstein, 1978; Rees and Dimmock, 1981b, 1982). These RNPs are found in both the

cytoplasm and the nucleus of infected cells (Krug, 1971) and appear able to cross the nuclear envelope in either direction (Hudson et al., 1978; Flawith and Dimmock, 1979).

The rapid entry of NP into the nucleus was also seen in oocytes injected with pTKNP. However, it is not clear if the average path length taken by an injected nuclear protein is similar to that taken by the same protein when it is expressed in oocytes via injected DNA; in the latter case it is possible that the encoding mRNA, and therefore translation, are concentrated around the nucleus (D. Drummond and A. Colman, unpublished data).

Although cyt-NP was toxic when injected into the nuclei of fresh oocytes its accumulation in the nucleus after injection into the cytoplasm shows that at equilibrium the NP in the oocyte cytoplasm is capable of entering the nucleus. Similarly the NP in the oocyte nucleus is capable of migrating back into the cytoplasm.

Due to the large energy reserves within the oocyte it is not feasible to use inhibitors to test for any energy requirement in the nuclear accumulation of NP. However, if the migration is an energy dependent process it should be virtually abolished by chilling to 4°C but be relatively unaffected if due to diffusion (Wu and Warner, 1971). In fact, when radiolabelled NP was injected into oocytes incubated at 4°C the rate of nuclear migration was decreased to less than 10% of that at 20°C. A similar result was obtained using nucleoplasmin (Dingwall et al., 1982) but, as discussed by these workers, interpretation is difficult since the low temperature is likely to affect many parameters.

Since no other influenza components are present in the oocytes injected with pTKNP the ability of the NP to accumulate in the nucleus is likely to be a property of the NP itself, while the similarity between the distribution of the cloned and authentic NP in BHK-21 cells suggests that the cloned protein is behaving normally. The influenza virus NP therefore appeared to be a good candidate for further analysis of nuclear accumulation and the following section describes the use of DNA recombinant methodology to produce mutant NP proteins in an attempt to identify those regions of the protein responsible for its nuclear accumulation.

SECTION V

THE NUCLEAR ASSOCIATION OF MUTANT NP PROTEINS

1. Introduction

The previous section described the nuclear accumulation of NP in Xenopus oocytes following its introduction into the oocytes as the protein itself or encoded in RNA or DNA. This accumulation appears to be a property of the protein itself, and since it occurs without any detectable modification of the protein it is likely to be controlled by a signal within the mature protein. This is the karyophilic signal (De Robertis et al., 1978). To establish the location of this signal I have used recombinant DNA methodology to delete various regions of the NP gene in pTKNP and then determined the location of the mutant NP proteins after injection of the deleted plasmids into Xenopus oocytes. This approach has previously been used successfully to locate other topogenic sequences (see 'General Introduction').

2. Results

a) Proteins lacking the carboxyl terminus of NP

Plasmids were constructed in which varying amounts of the region encoding the carboxyl terminus of NP was removed from pTKNP (see 'Methods B' and Table 14). After injection into oocytes each plasmid directed the synthesis of a protein of the predicted MW by SDS-PAGE (Figure 69). Several oocytes were enucleated and the isolated nuclear and cytoplasmic fractions immunoprecipitated with anti-NP antibody prior to analysis by SDS-PAGE (Figure 70). The concentration of the mutant NP proteins in each cellular compartment was determined and the resulting nuclear to cytoplasmic concentration ratios are summarised in Figure 71.

All of the proteins entered the nucleus, but only those containing the first 345 amino acids of the wild-type NP accumulated there; smaller proteins became evenly distributed between this compartment and the cytoplasm. One could argue that the reduced nuclear accumulation of the protein encoded by pTKNP Hind 1348 and the lack of accumulation of the smaller proteins is due either to a preferential degradation of these proteins in the nucleus or a decrease in their rates of nuclear entry. Both possibilities were investigated by performing a 48 h unlabelled chase on these oocytes after radiolabelling (Figure 72). The nuclear to cytoplasmic concentration ratios for these chased oocytes are shown in Table 15. Since there was no appreciable change in any of the ratios or in the total radioactivity incorporated into the mutant NPs it seems that the impairment of nuclear accumulation was due to the removal of all or part of a signal sequence necessary for such accumulation. These data also indicate that the mutant NP proteins had reached equilibrium

between the nucleus and the cytoplasm within the initial 6 h labelling period.

Table 14 Sequence and MW of mutant NP proteins encoded by plasmids lacking various amounts of the carboxyl terminus of the NP gene

Plasmid	Sequence at carboxyl terminus	Molecular weight
pTKNP Pvu 705	129 130 A T E L A D end GCA ACA GAG CTT GCT GAT TGA	15,450
pTKNP Pvu 1011	231 232 Q T E L A D end CAA ACA GAG CTT GCT GAT TGA	26,983
pTKNP Acc 1250	311 312 Q V end CAA GTA TAG	35,212
pTKNP Pvu 1295	326 327 S Q S L L I D end AGT CAG AGC TTG CTG ATT GAT TGA	37,487
pTKNP Hind 1345	344 345 L S L L I D end TAA AGC TTG CTG ATT GAT TGA	39,405
pTKNP Hae 1474	385 386 Y W E L A D end TAC TGG GAG CTT GCT GAT TGA	44,029
pTKNP	496 497 498 Y D N end TAC GAC AAT TAA	55,890

Amino acids are indicated by the single-letter code shown above the DNA sequence. The number indicates the position of the amino acid in the wild-type NP, the amino acids without numbers being encoded by the stop region.

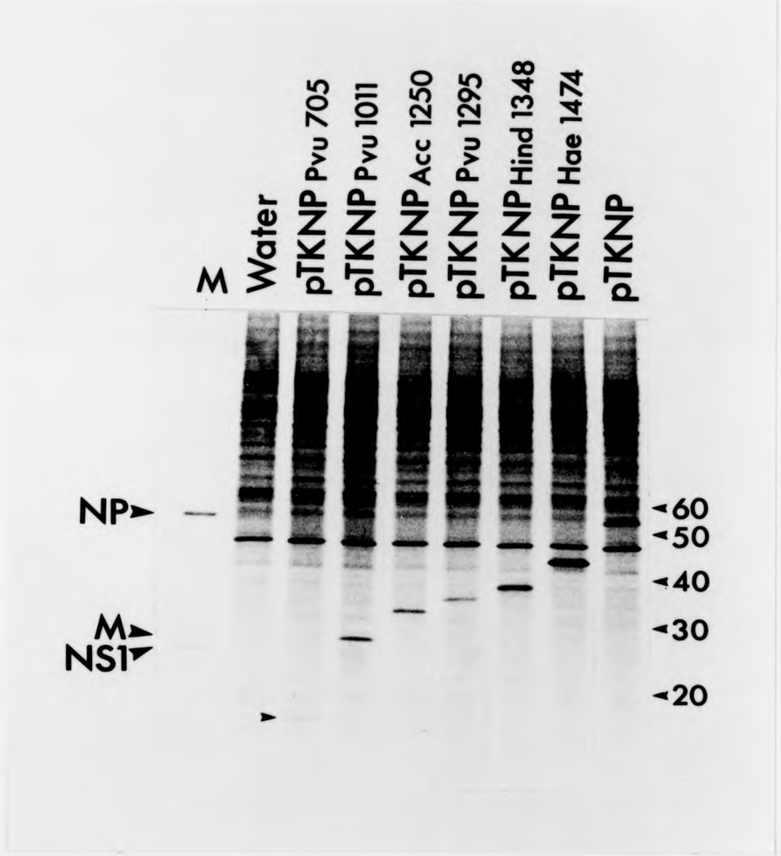


Figure 69 Synthesis of mutant NP proteins in oocytes injected with plasmids lacking various regions of the carboxyl terminus of the NP gene

After injection and radiolabelling the oocytes were homogenised and analysed by SDS-PAGE. The equivalent of one oocyte was loaded per track. Track M contains ^{35}S-methionine-labelled lysate from CEF cells infected with FP/R virus as marker.

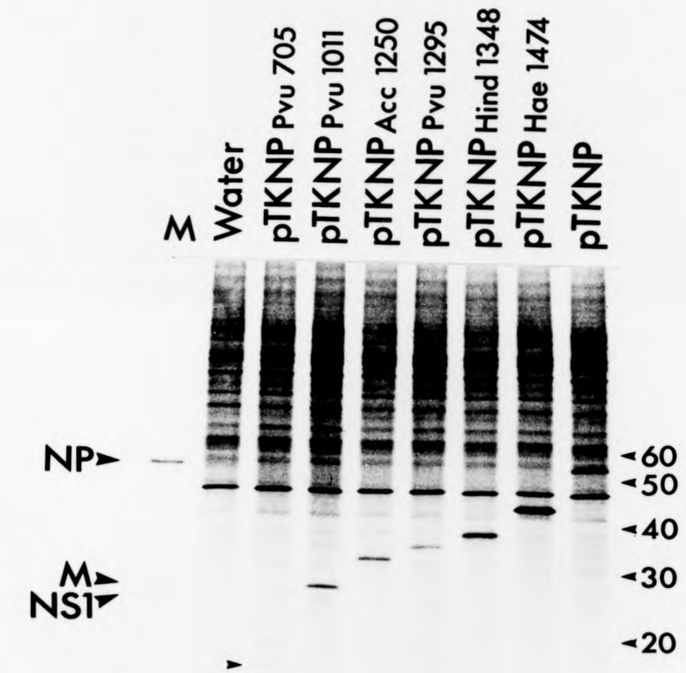


Figure 69 Synthesis of mutant NP proteins in oocytes injected with plasmids lacking various regions of the carboxyl terminus of the NP gene

After injection and radiolabelling the oocytes were homogenised and analysed by SDS-PAGE. The equivalent of one oocyte was loaded per track. Track M contains ^{35}S-methionine-labelled lysate from CEF cells infected with FP/R virus as marker.

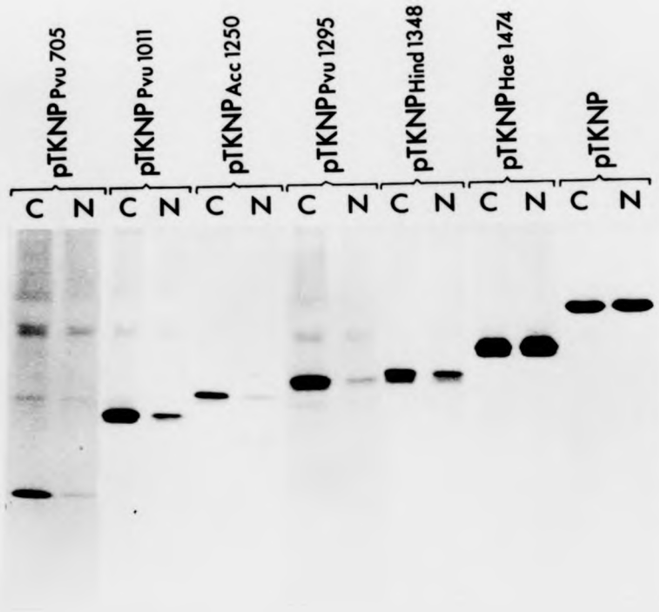


Figure 70 The nuclear association of mutant NP proteins lacking the carboxyl terminus of wild-type NP

Oocytes injected with the various plasmids were enucleated and the isolated nuclear and cytoplasmic fractions immunoprecipitated with anti-NP antibody prior to SDS-PAGE. The equivalent of one nucleus (N) and one cytoplasm (C) was loaded per track.

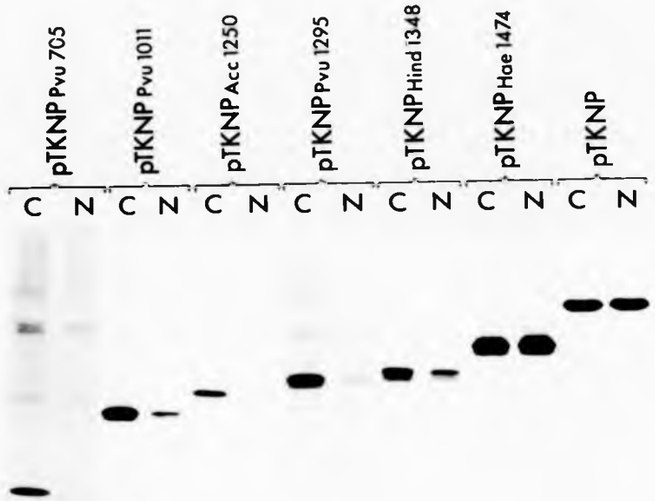


Figure 70 The nuclear association of mutant NP proteins lacking the carboxyl terminus of wild-type NP

Oocytes injected with the various plasmids were enucleated and the isolated nuclear and cytoplasmic fractions immunoprecipitated with anti-NP antibody prior to SDS-PAGE. The equivalent of one nucleus (N) and one cytoplasm (C) was loaded per track.

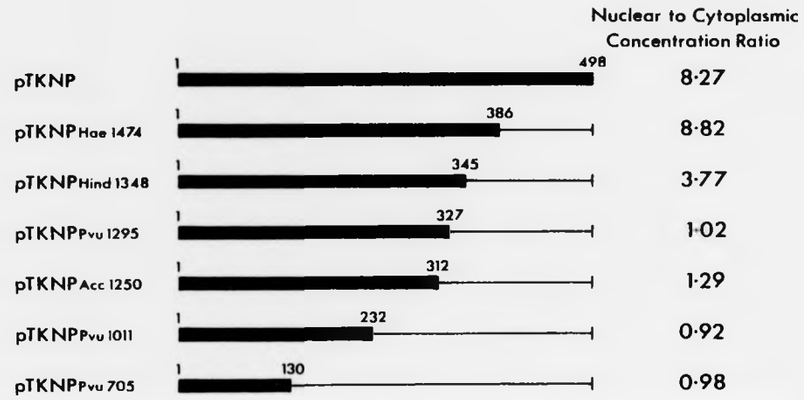


Figure 71 Nuclear to cytoplasmic concentration ratios of mutant NP proteins lacking the carboxyl terminus of wild-type NP

The thin line represents the region missing from the wild-type NP. The numbers refer to the positions of the terminal amino acids in NP. For convenience the amino acids encoded by the stop region are omitted.

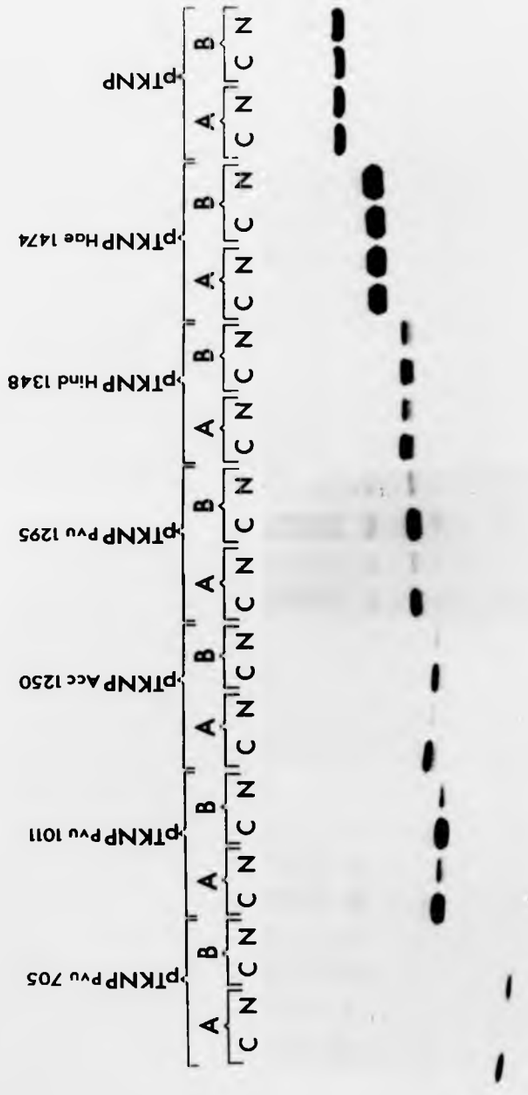


Figure 72 SDS-PAGE analysis of chased and unchased oocytes injected with plasmids lacking various amounts of the carboxyl terminus of the NP gene

After injection and radiolabelling half of the oocytes in each sample were enucleated while the remainder were incubated in unlabelled medium for 48 h prior to enucleation. The isolated nuclear (N) and cytoplasmic (C) fractions were immunoprecipitated with anti-NP antibody and analysed by SDS-PAGE. The equivalent of one nucleus and one cytoplasm was loaded per track. In each case sample A was obtained from the unchased oocytes while sample B was obtained from the chased oocytes.

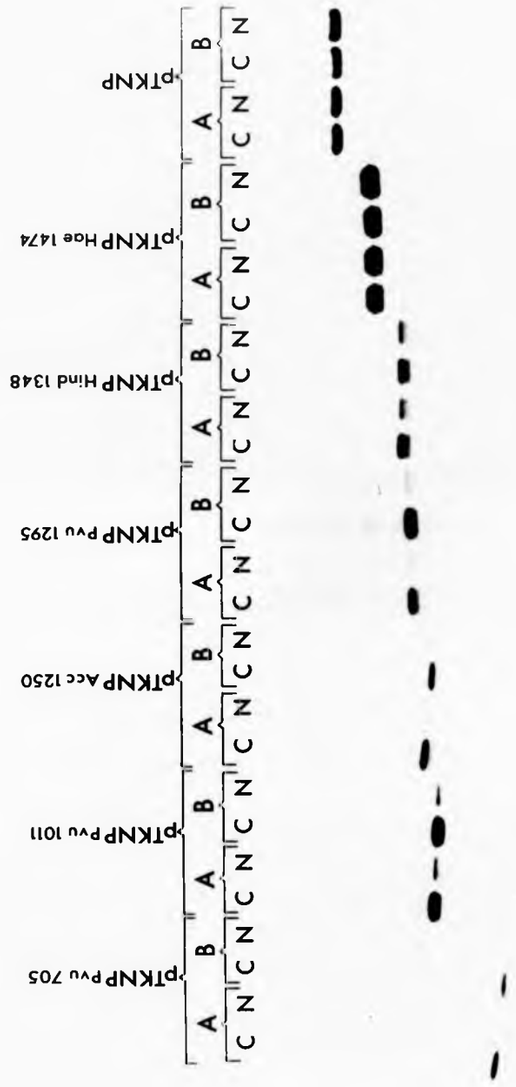


Figure 72 SDS-PAGE analysis of chased and unchased oocytes injected with plasmids lacking various amounts of the carboxyl terminus of the NP gene

After injection and radiolabelling half of the oocytes in each sample were enucleated while the remainder were incubated in unlabelled medium for 48 h prior to enucleation. The isolated nuclear (N) and cytoplasmic (C) fractions were immunoprecipitated with anti-NP antibody and analysed by SDS-PAGE. The equivalent of one nucleus and one cytoplasm was loaded per track. In each case sample A was obtained from the unchased oocytes while sample B was obtained from the chased oocytes.

Table 15 The nuclear to cytoplasmic concentration ratios for unchased and chased oocytes injected with plasmids lacking various amounts of the carboxyl terminus of the NP gene

Plasmid	Nuclear to cytoplasmic concentration ratio	
	Unchased	Chased
pTKNP	8.27	8.41
pTKNP Hae 1474	8.82	9.12
pTKNP Hind 1348	3.77	4.10
pTKNP Pvu 1295	1.02	1.09
pTKNP Acc 1250	1.29	1.02
pTKNP Pvu 1011	0.92	1.11
pTKNP Pvu 705	0.98	1.08

b) Proteins lacking internal regions of NP

Plasmids were constructed in which regions were removed from within the NP coding sequence of pTKNP (see 'Methods B'). The predicted sequences of the mutant NPs are summarised in Table 16. After injection into oocytes each plasmid directed the synthesis of a protein of the predicted MW by SDS-PAGE (Figure 73). Several oocytes were enucleated and the isolated nuclear and cytoplasmic fractions immunoprecipitated with anti-NP antibody prior to analysis by SDS-PAGE (Figure 74). The nuclear to cytoplasmic concentration ratios of these proteins are summarised in Figure 75. All the mutant proteins accumulated in the nucleus, but the mutant lacking amino acids 255 to 339 did so to a much lesser extent than the others.

Table 16 Sequence and MW of mutant NP proteins encoded by plasmids lacking sequences from within the NP gene

Plasmid	Sequence at ligation point	Molecular weight
pTKNP Bam 577/793	86 87 160 161 G K D P GGG AAG GAT CCC	47,512
pTKNP Pvu 705/1011	129 130 233 234 A T A A GCA ACA GCT GCA	44,357
pTKNP Bgl 1078/1333	253 254 340 341 I E D L ATC GAA GAT CTA	46,630
pTKNP Imp 1295	1 2 328 329 M A L V ATG GCG CTG GTG	19,164

Amino acids are indicated by the single-letter code shown above the DNA sequence. The number indicates the positions of the amino acid in the wild-type NP.

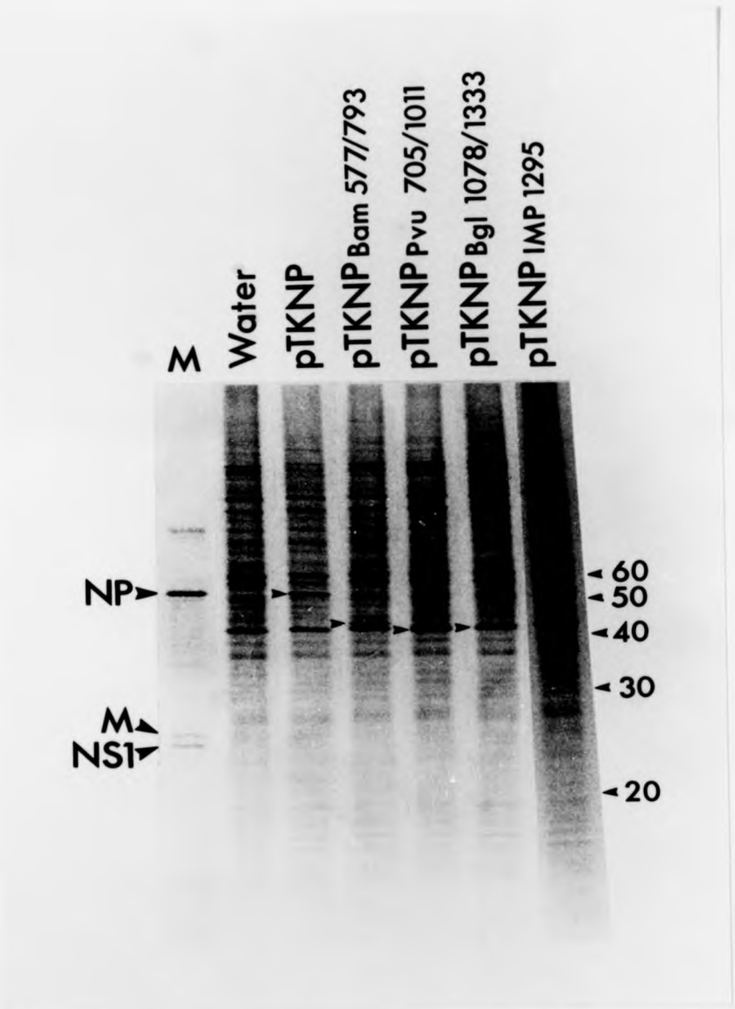


Figure 73 Synthesis of mutant NP proteins in oocytes injected with plasmids lacking sequences from within the NP gene

After injection and radiolabelling the oocytes were homogenised and analysed by SDS-PAGE. The equivalent of one oocyte was loaded per track. Track M contains ^{35}S-methionine-labelled lysate from CEF cells infected with FP/R virus as marker.

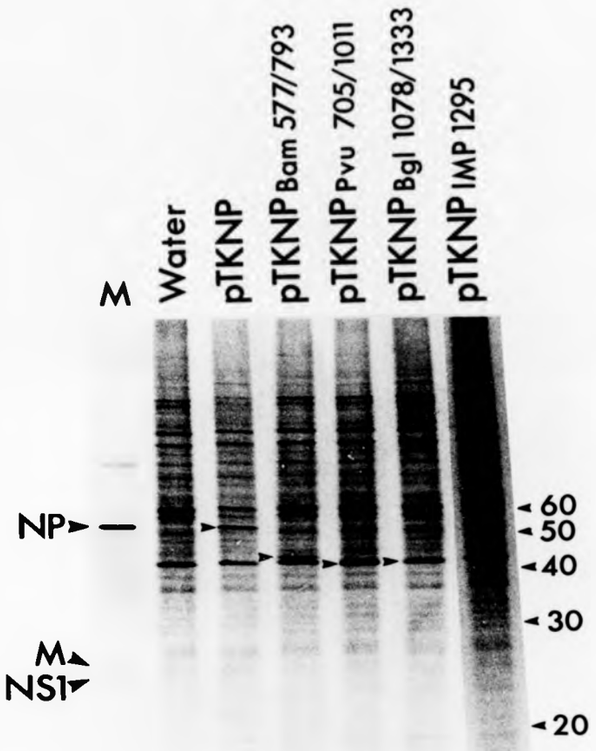


Figure 73 Synthesis of mutant NP proteins in oocytes injected with plasmids lacking sequences from within the NP gene

After injection and radiolabelling the oocytes were homogenised and analysed by SDS-PAGE. The equivalent of one oocyte was loaded per track. Track M contains ^{35}S-methionine-labelled lysate from CEF cells infected with FP/R virus as marker.

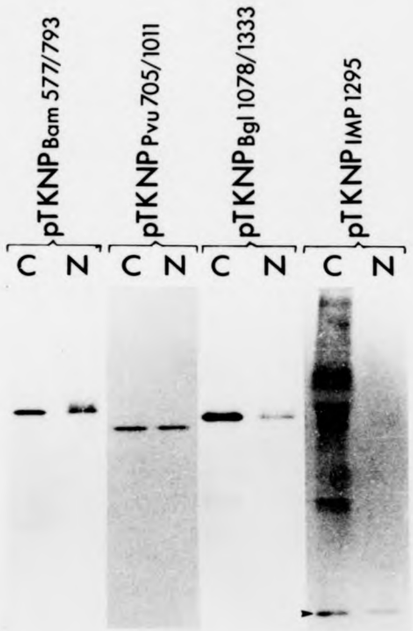


Figure 74 The nuclear association of mutant NP proteins lacking internal regions of wild-type NP

Oocytes injected with the various plasmids were enucleated and the isolated nuclear and cytoplasmic fractions immunoprecipitated with anti-NP antibody prior to SDS-PAGE. The equivalent of one nucleus (N) and one cytoplasm (C) was loaded per track.

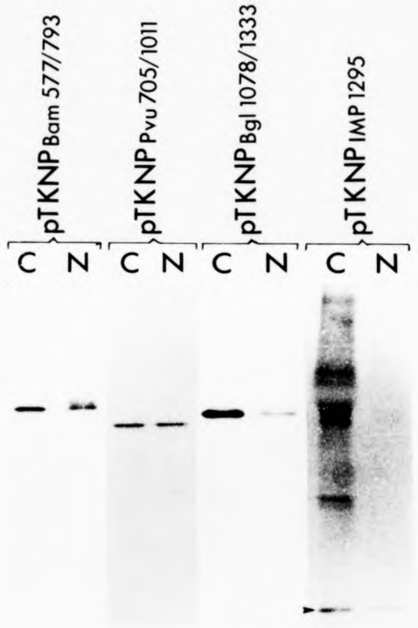


Figure 74 The nuclear association of mutant NP proteins lacking internal regions of wild-type NP

Oocytes injected with the various plasmids were enucleated and the isolated nuclear and cytoplasmic fractions immunoprecipitated with anti-NP antibody prior to SDS-PAGE. The equivalent of one nucleus (N) and one cytoplasm (C) was loaded per track.

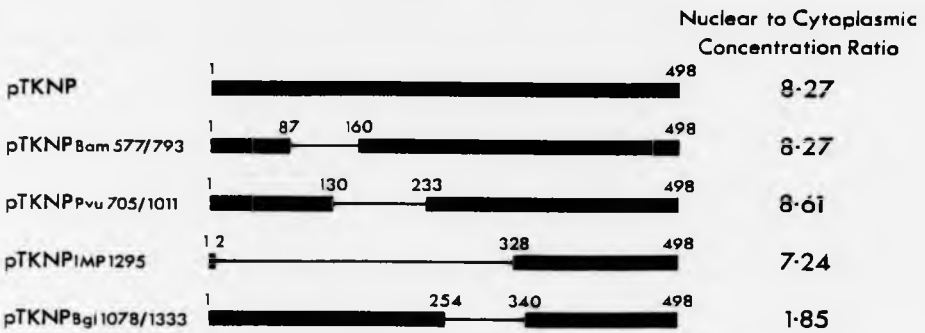


Figure 75 Nuclear to cytoplasmic concentration ratios of mutant NP proteins lacking internal regions of wild-type NP

The thin line represents the region missing from the wild-type NP. The numbers refer to the positions of the terminal amino acids in NP.

c) Proteins lacking the amino terminus of NP

Plasmids were constructed in which varying amounts of the region encoding the amino terminus of NP was removed from pTKNP (see 'Methods B' and Table 17). After injection of these plasmids into oocytes no NP-related proteins were detected even upon immunoprecipitation with anti-NP antibody.

Table 17 Sequence and MW of mutant NP proteins encoded by plasmids lacking various amounts of the amino terminus of the NP gene

Plasmid	Sequence at amino terminus	Molecular weight
pTKNP	1 2 M A AAAATC ATG GCG	55,890
pTKNP Hind 1348C	371 372 M D GAAAAC ATG GAT	14,262
pTKNP Met 136	136 137 M M ATCCAC ATG ATG	40,407
pTKNP Met 238	238 239 M M AGGGCA ATG ATG	28,856
pTKNP Met 331/S	331 332 M A GTGTGG ATG GCA	18,564
pTKNP Met 331/L	331 332 M A GTGTGG ATG GCA	18,564

Amino acids are indicated by the single-letter code shown above the DNA sequence. The number indicates the position of the amino acid in the wild-type NP. The first methionine should act as the initiator in the mutant NP proteins.

3. Discussion

a) Proteins lacking the amino terminus of NP

Two reasons can be envisaged for the lack of detectable protein products following the injection of plasmids lacking the amino terminus of the NP gene; either the products are unstable and are rapidly degraded by the oocytes or no proteins are produced or are produced at very low levels. Since the protein NP IMP 1295 was detected whereas no NP related protein was detected in oocytes injected with either pTKNP Met 331/S or pTKNP Met 331/L (which should direct the synthesis of a protein very similar to NP IMP 1295) it seems that the latter possibility is more likely.

The lack of transcriptional analysis means it is not possible to conclude whether or not the lack of detectable protein production is the result of inefficient transcription or inefficient translation. However, since each mutant plasmid has a transcriptional promotor region identical to pTKNP it is more likely that the problem is one of inefficient translation. Indeed a similar deletion of a plasmid encoding a secretory protein does produce a transcript which is transported to the cytoplasm but is not translated (L. Tabe, unpublished data). Possible explanations as to why such transcripts may not be efficiently translated are revealed by the work of Marilyn Kozak. The analysis of sequences from over 200 eukaryotic mRNAs of both viral and cellular origin has enabled Kozak to identify several conserved (or semi-conserved) features of these transcripts (Kozak, 1981a, 1983, 1984a).

i) Translation is initiated at the first AUG triplet

In vivo initiation of translation of cytoplasmic eukaryotic mRNAs occurs exclusively at AUG codons although in heterologous in vitro systems in which the ribosomes are derived from E. coli GUG has also been osberved to initiate translation (Raj Bhandary and Ghosh, 1969). This is in contrast to both prokaryotes where GUG (Wezenbeek and Schoenmakers, 1979), UUG (Thach et al., 1966; Mackie, 1981), AUU (Sacerdot et al., 1982), AUA (Johnston and Roth, 1981) and ACG (Thach et al., 1966) can also initiate translation and mammalian mitochondrial mRNAs where initiation of translation usually occurs at the triplets AUU and AUA (Bibb et al., 1981; Montoya et al., 1981).

In about 95% of eukaryotic mRNAs the initiation of translation occurs at the AUG triplet that is nearest to the 5' end of the transcript (Kozak, 1981a, 1983, 1984a). This first AUG rule holds true for mRNAs with very long 5' leaders (Gallwitz et al., 1981; Ingolia and Craig, 1981; Derynck et al., 1982; Noda et al., 1982). In those cases where one or more AUG triplets occur upstream from the recognised or functional initiation codon (see, for example, Hendy et al., 1981; Clerx-van Haaster et al., 1982; Yoo et al., 1982) the upstream AUGs are called 'non-functional' since there is as yet no evidence that ribosomes recognise these sites. Theory however (see below) predicts that ribosomes should initiate at the upstream AUG triplets as well as at the AUG triplet that heads the long open reading frame.

Circumstantial evidence (reviewed by Kozak, 1978, 1980,1981b, 1982B) supports the hypothesis that 40S ribosomal subunits bind initially at, or near, the 5' end of the mRNA and then migrate to the initiation

codon. In the earliest version of this 'scanning model' when the 40S subunit encountered the first AUG triplet a 60S subunit would attach and translation would begin. This would mean that initiation of translation would always occur at the 5'-proximal AUG. As mentioned above, however, there are examples of mRNAs where the functional AUG codon is not the first in the transcript. Inspection of the nucleotide sequences flanking the apparently non-functinal upstream AUG triplets led to a modified vesion of the scanning model (Kozak, 1981b). The current hypothesis is that the 40S ribosomal subunits bind at the 5' end of the mRNA and migrate inwards as before but the efficiency with which an AUG triplet is recognised depends on the sequence around the AUG (this is discussed in more detail below). According to the modified scanning model, if the first AUG triplet occurs in the optimal sequence context 40S subunits will initiate uniquely at that site; however, if the first AUG triplet occurs in a less favourable context some 40S subunits will stop and initiate there, whereas some will bypass that site and initiate farther downstream. It is therefore possible for a single eukaryotic mRNA to direct the synthesis of two proteins. Although a few such mRNAs have been identified (Table 18) they are rare and normally the 5'-proximal AUG triplet occurs in a favourable sequence context.

Since the 5'-proximal AUG is the first to be inspected most of the mutant NP plasmids were constructed so that the first AUG was in the correct reading frame, i.e. encoded an internal methionine in the wild-type NP.

Table 18 Messenger RNAs which direct the synthesis of more than one protein

Simian virus 40 late 19S mRNA[a]	Piatak et al., 1979
Adenovirus Elb mRNA[b]	Bos et al., 1981
Bunyavirus s-RNA[b]	Bishop et al., 1982
Herpes simplex virus thymidine kinase mRNA[c]	Preston and McGeoch, 1981
pTKOV- mRNA[d]	Tabe et al., 1984

a) The second protein is a shortened version of the first (VP2 and VP3)

b) The initiation codons are in different reading frames and two unrelated proteins are produced.

c) This is different to the situation which gave ovalbumin and an amino-terminally extended ovalbumin after injection of pTKOV into oocytes (see 'Results', Section III and Tabe et al., 1984).

d) see 'Results', Section III.

ii) Distance from the 5' end of the transcript to the AUG initiation codon

The length of the untranslated sequence at the 5' end of eukaryotic mRNAs varies from 3 (Kelley et al., 1982) to 742 (Kitamura et al., 1981) nucleotides. Exceptionally short and long leaders are rare and are usually confined to viral messengers (Lebowitz and Weissman, 1979; Kitamura et al., 1981; Swanstrom et al., 1982) or to the transcripts of unusually interesting cellular genes. Heat shock proteins, for example, have very long leader sequences (Holmgren et al., 1981; Ingolia and Craig, 1981) and this has invited a great deal of speculation about the possible role of the 5' non-coding region in the regulated expression of these genes. Most cellular mRNAs however have leader sequences of between 40 and 80 nucleotides (Kozak, 1982b).

From studies of the transcription of the HSV thymidine kinase promotor in Xenopus oocytes, when present with the complete thymidine kinase gene (McKnight and Gavis, 1980; McKnight et al., 1981) or as part of a plasmid (Krieg et al., 1984) (see 'Results', Section III), it is possible to locate the site of initiation of transcription to nucleotides 195/196 in pTKNP (see Figure 58). Table 19 shows the number of nucleotides between the transcriptional initiation site and the first in phase ATG triplet in the plasmids lacking various regions of the amino terminus of the NP gene. There are no exceptionally short or long leader sequences and it is therefore unlikely that inefficient translation is due to the length of the 5' non-coding region.

Table 19 Length of the 5' non-coding sequence in the transcripts predicted from plasmids lacking various amounts of the amino terminus of the NP gene

Plasmid	Number of nucleotides from cap site to first in phase AUG
pTKNP	121
pTKNP Hind 1348C	138
pTKNP Met 136	78
pTKNP Met 238	78
pTKNP Met 331/S	73
pTKNP Met 331/L	119

iii) Initiator AUG codons occur in conserved sequence contexts

Initiator AUG codons do not occur in random sequence environments. The most conspicuous conserved feature is the presence of a purine, most often an adenosine, in position -3, i.e. three nucleotides upstream from the initiator codon. As illustrated in Table 20, 70% of the analysed mRNAs have A in that position, 18% have G and only 3% have a pyrimidine (Kozak, 1984a). This preference is not found around AUG triplets that code for methionine at internal positions in polypeptide chains (Table 20). The direct demonstration of the importance of a purine at position -3 has been obtained using in vitro binding experiments with various AUG-containing oligonucleotides (Kozak, 1981b) and, more recently, site directed mutagenesis of the AUG initiation codon in a cloned pre-proinsulin gene (Kozak, 1984b). With the pre-proinsulin gene the replacement of a pyrimidine at position -3 with a guanosine caused a five-fold increase in the efficiency of translation while replacement with an adenosine caused a 15-fold increase. This effect has also been demonstrated indirectly by the insertion of AUG codons upstream of the functional initiation codon in a vector expressing the gene encoding the surface antigen of Hepatitis B virus (Liu et al., 1984). Taken together these results suggest that, with all other factors being equal, the efficiency of an AUG triplet to act as initiator for translation decreases in the order ANNAUG, GNNAUG, YNNAUG where Y is a pyrimidine and N is either a pyrimidine or a purine.

The relevant AUG triplets in the transcripts proposed from the various plasmids have the sequence contexts shown in Table 21. In three of the five mutants there is a pyrimidine at position -3 while in pTKNP Met 238 there is a guanosine. It is therefore possible that initiation of

Table 20 Sequence context around AUG triplets in eukaryotic mRNAs (modified from Kozak, 1983)

Sequence[1]	Percentage of mRNAs containing sequence as: Functional initiator codon[2]	Internal AUG codon[3]
ANNAUG	79	30
GNNAUG	18	35
YNNAUG	3	35

1) N indicates any nucleotide while Y indicates a pyrimidine.

2) From 180 mRNAs identified in Kozak, 1981b, 1982a.

3) From 24 mRNAs identified in Kozak, 1982a.

Table 21 Sequence context around the first in phase AUG triplet in the transcripts predicted from plasmids lacking various amounts of the amino terminus of the NP gene

Plasmid	Sequence around the first in phase AUG
pTKNP	ANNAUG
pTKNP Hind 1348C	ANNAUG*
pTKNP Met 136	CNNAUG
pTKNP Met 238	GNNAUG
pTKNP Met 331/S	UNNAUG
pTKNP Met 331/L	UNNAUG

* This is preceeded by an upstream, out of phase, AUG triplet with the sequence context 5'-CNNAUG-3'.

translation does occur at these codons but does so at an efficiency that is too low to allow the detection of any product. The present hypothesis of the scanning model for the initiation of translation (see above) predicts that if the 5'-proximal AUG occurs in an unfavoured environment then initiation will also occur at the second AUG. In each case this second AUG occurs within two nucleotides of the first and it is possible that initiation at the second triplet is inhibited by the binding of ribosomes to the first. The third AUG triplet in each case has an adenosine in psoition -3 but is in the wrong reading frame.

The plasmid pTKNP Hind 1348C is different to the others in that the first in phase AUG triplet is preceeded by another AUG which is in the incorect reading frame. As described in 'Methods B' initiation of translation at this 5'-proximal AUG would produce a peptide containing 13 amino acids (Figure 30). This upstream AUG has a pyrimidine at position -3 and so would be expected to initiate translation inefficiently. The lack of a detectable protein product initiated at the second AUG even though it has an adenosine at position -3 suggests that initiation at this triplet is also inefficient. The effect of an upstream non-functional AUG triplet on the efficiency of translation from a functional AUG is discussed in more detail by Liu et al. (1984).

Although no other position around the AUG initiation codon is as highly conserved as position -3, the distribution of nucleotides is markedly non-random in every position from -1 through to -6, and perhaps beyond (Kozak, 1981b, 1984a). This includes the presence of guanosine at position +4 and, less frequently, cytosine in positions -1, -2, -4 and -5. Since these positions are less conserved than position -3 and have

not been directly demonstrated to contribute to the recognition of eukaryotic initiation sites I will not include them in this discussion.

It should be noted that sequences other than those discussed above seem to be involved in the recognition process. This was demonstrated by the insertion of AUG triplets uptream of the functional AUG vector expressing the surface antigen of Hepatitis B virus (Liu et al., 1984). The insertion of a synthetic AUG with an adenosine at position -3 and a guanosine at position +4 decreased the level of translation from the functional AUG but the inhibition of translation was increased by the insertion of an 'authentic' AUG.

In the above discussion I have proposed a possible explanation for the lack of a detectable protein product from plasmids lacking the amino-terminal region of the NP gene. Although this explanation is feasible there is no direct evidence to support it.

b) Nuclear association of the mutant NP proteins

Although all of the mutant NP proteins entered the oocyte nuclei and at least became evenly distributed between this compartment and the cytoplasm only some mutants accumulated there. Since the loss of nuclear accumulation was associated with the loss of amino acids 327 to 345 of the wild-type NP it appears that the information necessary for the nuclear accumulation of this protein is contained wholly or partly within this region. By definition therefore this region contains the karyophilic signal itself or is responsible for maintaining the integrity of a signal region elsewhere in the protein. The amino-

terminal limit is set by the protein encoded by pTKNP IMP 1295 which basically contains amino acids 328 to 498, and accumulated in the nucleus to a similar extent to wild-type NP. The carboxyl-terminal limit is less well defined and based principally on the nuclear accumulation of the protein containing the first 345 amino acids of NP. However, the fact that this mutant accumulated to a lesser extent than the wild-type NP suggests it may not contain all of the karyophilic signal. These observations on the accumulation of different mutant NP proteins whose only common feature is the segment of amino acids 327 to 345 argue against the indirect signalling role of this region described above.

SECTION VI

THE NUCLEAR ASSOCIATION OF NP-GLOBIN FUSION PROTEINS

1. Introduction

The above use of deletion mutants suggests that the information necesary for the nuclear accumulation of NP resides wholly or partly within the region from amino acids 327 to 340 since mutant proteins lacking this region have a reduced ability to accumulate in the nucleus. This strategy, however, suffers from the criticism that the removal of large regions of the NP may cause gross changes in its conformation which could impair nuclear accumulation. The presence of a karyophilic signal in a region of the NP can be tested positively by constructing fusion proteins containing various regions of the NP and a protein which ordinarily does not accumulate in nuclei. Accumulation in the nucleus of the fusion product would then indicate that the NP region present in the fusion protein contained the information necessary for this process. The same strategy has been used to study signal sequences in prokaryotes (Silhavy et al., 1977; Bedoulle et al., 1980; Emr et al., 1980), in in vitro systems (Lingappa et al., 1984) and in eukaryotes (Tabe et al., 1984).

An important consideration in such experiments is the choice of protein to be used in the fusion. Globins are ideal candidates since they exhibit no affinity for membranes either in vitro (Meek et al., 1982) or in vivo in Xenopus oocytes (Zehavi-Willner and Lane, 1977), and have recently been used successfully in fusions involving secretory proteins (Lingappa et al., 1984; Tabe et al., 1984).

2. Results

The plasmid pTKG contains the full length cDNA of chimpanzee α_1-globin under the control of the eukaryotic expression elements in the vector pTK_2 (see 'Methods B'). Upon injection into oocytes this plasmid directed the synthesis of a protein of approximately 12,500 daltons which was precipitated by anti-globin antibody and was not detected in the nucleus (Figure 76).

Four plasmids were constructed in which the globin cDNA was fused to various amino-terminal regions of the NP cDNA (see 'Methods B'). The sequence and MWs of proteins predicted from these plasmids are summarised in Table 22, and after injection into oocytes such proteins were produced. Injected oocytes were immunoprecipitated with anti-globin antibody while the isolated nuclear and cytoplasmic fractions were immunoprecipitated with anti-NP antibody prior to analysis by SDS-PAGE (Figure 76). Each protein was precipitated by both antibodies. The concentration of the proteins in each cellular compartment was determined and used to calculate the nuclear to cytoplasmic concentration ratios which are summarised in Figure 77. Although all the fusion proteins entered the oocyte nucleus only the protein produced from pTKNPG accumulated there.

Table 22 Properties of the NP-globin fusion proteins

Plasmid	Sequence at fusion point	Molecular weight
pTKNP130G	129 130 1 2 A T G G S M V GCA ACA GGG GGA TCC ATG GTG NP Globin	27,700
pTKNP232G	231 232 1 2 Q T G G S M V CCA ACA GGG GGA TCC ATG GTG NP Globin	39,200
pTKNP312G	311 312 1 2 Q V W G S M V CAA GTA TGG GGA TCC ATG GTG NP Globin	48,000
pTKNPG	Not known	65,000

The NP and globin sequences at the fusion joints are underlined and labelled. The DNA that is not underlined is from M13 mp10. Amino acids are indicated by the single-letter code while the number above an amino acid indicates its position in the respective wild-type protein. Although not known precisely it has been estimated that pTKNPG has the first 460 amino acids of NP. The molecular weights were calculated using the known amino acid compositions of the NP and linker regions and a value of 12,500 daltons for the globin region which was estimated from the migration of the protein produced in pTKG-injected oocytes.

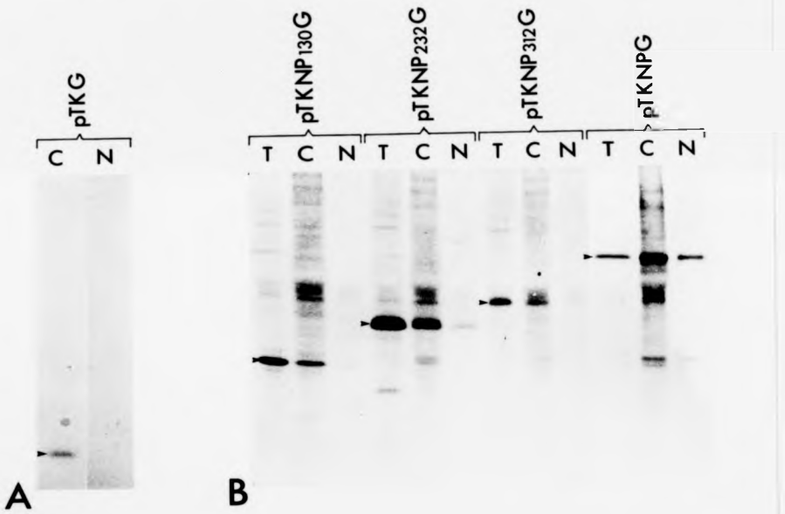


Figure 76 Injection into oocytes of plasmids encoding the NP-globin fusion proteins

Total (T), cytoplasmic (C) and nuclear (N) fractions were obtained after injecting oocytes with plasmids encoding α_1-globin and NP-globin fusion proteins. The fractions of the oocytes injected with pTKG were immunoprecipitated with anti-globin antibodies (section A). For oocytes injected with plasmids encoding the NP-globin fusion proteins the cytoplasmic and nuclear fractions were immunoprecipitated with anti-NP antibody while the total oocyte fraction was immunoprecipitated with anti-globin antibody (section B). The equivalent of one oocyte or one oocyte fraction was loaded per track.

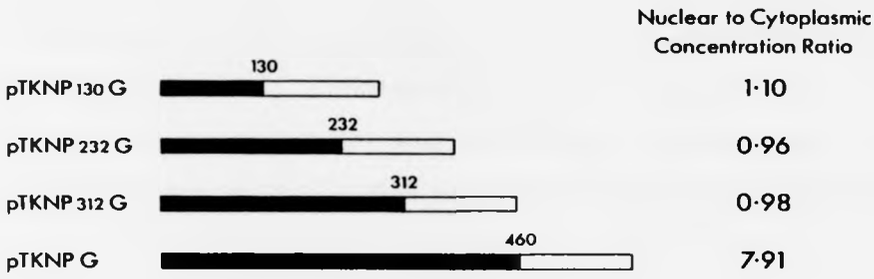


Figure 77 Nuclear to cytoplasmic concentration ratios of the NP-globin fusion proteins

The solid box represents the NP region of the fusion protein and the open box the globin region. The numbers refer to the positions of the terminal amino acids in the wild-type NP. For convenience the amino acids encoded by the linker region are omitted.

3. Discussion

The nuclear associations of the four NP-globin fusion proteins mimicked those of the NP fragments they contained (see Figure 78). The fusion protein containing amino acids 327 to 345 of NP attained a nuclear to cytoplasmic ratio of approximately 8 while the fusions lacking this region became evenly distributed between the cytoplasm and the nucleus. This localisation of the NP karyophilic signal to amino acids 327 to 345 utilises previous results with the NP deletion mutants since from the use of fusion proteins alone it can only be concluded that this signal resides in the region from amino acids 312 to 460. A more stringent testing of this proposed location would be to construct fusion proteins containing fragments of NP which more closely delineate the 327 to 345 amino acid region, and ultimately to construct a fusion protein containing all of the globin sequence and only amino acids 327 to 345 of NP. A lack of time prevented this further analysis.

Globin could not be detected in oocyte nuclei, but when fused to the first 130 amino acids of NP the resulting fusion protein was capable of entering the nucleus and became evenly distributed between this compartment and the cytoplasm. This could mean that the first 130 amino acids of NP contains all the information necessary for ~~transport~~ entry into the nucleus (see 'General Discussion').

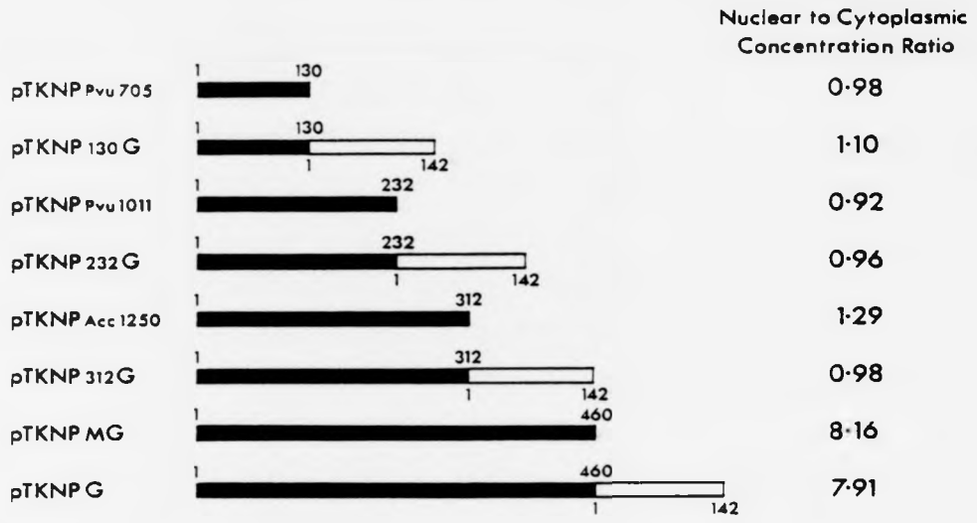


Figure 78 Nuclear to cytoplasmic concentration ratios of the NP-globin fusion proteins and the relevant mutant NP proteins

The solid box represents the NP region and the open box the globin region. The numbers refer to the positions of the terminal amino acids in the wild-type proteins. For convenience the amino acids encoded by the stop region and the linker regions are omitted. The plasmid encoding the first 460 amino acids of NP (pTKNPMG) was a biproduct of the construction of pTKNPG (see 'Methods B'). Both plasmids give the same pattern of restriction enzyme digestion but in pTKNPMG the NP and globin encoding regions are in different reading frames. After injection into oocytes pTKNPMG directed the synthesis of a protein of 52,000 daltons which was precipitated by anti-NP antibody, but was not precipitated by anti-globin antibody (see Figure 79).

Figure 79 Nuclear accumulation of the protein encoded by the plasmid pTKNPMG

Oocytes injected with pTKNPMG were enucleated and the isolated nuclear (N) and cytoplasmic (C) fractions immunoprecipitated with anti-NP antibody prior to SDS-PAGE. Total oocytes (T) were immunoprecipiated with anti-globin antibody. The equivalent of one oocyte or one oocyte fraction was loaded per track. This result is included here rather than in the section describing results obtained using other amino-terminal fragments of NP since the sequence at the carboxyl terminus of the protein is not known with any certainty.

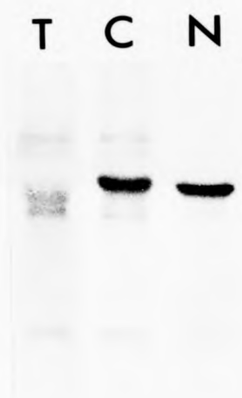


Figure 79 Nuclear accumulation of the protein encoded by the plasmid pTKNPMG

Oocytes injected with pTKNPMG were enucleated and the isolated nuclear (N) and cytoplasmic (C) fractions immunoprecipitated with anti-NP antibody prior to SDS-PAGE. Total oocytes (T) were immunoprecipiated with anti-globin antibody. The equivalent of one oocyte or one oocyte fraction was loaded per track. This result is included here rather than in the section describing results obtained using other amino-terminal fragments of NP since the sequence at the carboxyl terminus of the protein is not known with any certainty.

GENERAL DISCUSSION

This final discussion expands on some of the points raised during this thesis and discusses the relationship of this work to that of others.

1. Current models for the nuclear accumulation of proteins

Two alternative models are commonly considered to explain the nuclear accumulation of certain proteins (Dingwall et al., 1982; De Robertis, 1983; Hall et al., 1984). The first envisages the free diffusion of all proteins into the nucleus with the subsequent retention of nuclear proteins by their binding to non-diffusible nuclear elements, while the second suggests that the accumulation of nuclear proteins is due to their selective transport across the nuclear envelope. Since nuclear accumulation is selective and occurs post-translationally with no apparent modification of the proteins (Gurdon, 1970; Bonner, 1975a, 1975b; De Robertis et al., 1978; De Robertis and Black, 1981; Dabauvalle and Franke, 1982; Dingwall et al., 1982) it has been proposed that nuclear proteins contain within their mature molecular structure a signal sequence (or other property such as conformation) which controls their accumulation (De Robertis et al., 1978). In the first model this karyophilic signal would be responsible for the nuclear retention of the protein while in the second model its interaction with the nuclear envelope would allow selective transport of the protein into the nucleus. Experimental evidence however suggests that neither model is wholly correct. If all proteins entered nuclei by free diffusion then, assuming a functional nuclear pore radius of 45 Å for the Xenopus oocyte nucleus (Paine et al., 1975) one would predict that, for these

nuclei, proteins with a MW >70,000 would be excluded while proteins smaller than this would enter at rates which are dependent upon the size of the protein. Whereas non-nuclear proteins obey these rules after injection into Xenopus oocytes (Gurdon, 1970; Bonner, 1975a, 1975b), nuclear proteins do not. For instance, many are considerably larger than 70,000 daltons (De Robertis et al., 1978; Mills et al., 1980; Feldherr et al., 1983) and smaller proteins enter at rates which are faster than predicted (Gurdon, 1970; Bonner, 1975a, 1975b). Evidence against proteins accumulating by selective transport across the nuclear envelope is based on experiments which show that the puncture (Feldherr and Pomerantz, 1978; Feldherr and Ogburn, 1980) or removal (cited in De Robertis, 1983) of the nuclear envelope has little effect on the ability of the nucleus to accumulate the majority of nuclear proteins. On the basis of such previously published data and the results with influenza virus NP I propose a third model for the nuclear accumulation of proteins which combines certain aspects of both the previous models. I suggest that both selective (or more accurately mediated) transport and selective binding play a role in nuclear accumulation and that it is possible that these two activities are controlled by different regions of the protein.

2. Localisation of the NP karyophilic signal

Although all of the mutant NP proteins entered the oocyte nucleus and at least became equally distributed between this compartment and the cytoplasm only some mutants accumulated there. The loss of nuclear

accumulation was associated with the loss of amino acids 327 to 345 of the wild-type NP suggesting that the information necessary for the nuclear accumulation of this protein is contained wholly or partly within this region. By definition therefore, this region contains the karyophilic signal (De Robertis et al., 1978).

The results obtained with the NP-globin fusion proteins are consistent with this proposed location of the karyophilic signal since the fusion protein containing this region accumulated in the nucleus while those lacking this region did not accumulate but became evenly distributed between this compartment and the cytoplasm.

The identification of the NP karyophilic signal does not reveal how it controls the nuclear accumulation of this protein. It could act at the nuclear envelope by controlling a transport process which leads to accumulation or it could help to retain the protein in the nucleus after transport. By analogy to the majority of nuclear proteins, which accumulate in nuclei in the absence of the nuclear envelope, it could be suggested that the accumulation of NP is likely to result from its binding to a non-diffusible nuclear substrate.

3. Sequence homologies between nuclear proteins

The amino acid sequence of wild-type NP from residues 325 to 350 is shown in Figure 80. Although there is no tract of basic residues similar to that which may be important in the nuclear accumulation of

Figure 80 Amino acid sequence of wild-type NP from residues 325 to 350

325					330					335		
Lys	Ser	Gln	Leu	Val	Trp	Met	Ala	Cys	Asn	Ser	Ala	Ala
+			H	H		H						

	340					345					350
Phe	Glu	Asp	Leu	Arg	Val	Leu	Ser	Phe	Ile	Arg	Gly	Thr
H	-	-	H	+	H	H		H	H	+		

Amino acids are indicted by the three-letter code and are shown as being basic (+), acidic (-) or hydrophobic (H). The number above the amino acid indicates its position in NP.

the SV40 large-T antigen (Kalderon et al., 1984) there is a sequence similar to one within the proposed karyophilic signal-containing region of the yeast ∝2 protein (Hall et al., 1984). The ∝2 sequence has two basic amino acids flanking three hydrophobic residues while the NP sequence has two basic amino acids flanking five residues, four of which are hydrophobic. Whether these sequences have any importance in nuclear accumulation will only become evident after analysis of mutants altered in these residues. The ∝2 sequence is also found in certain other yeast nuclear proteins (see Hall et al., 1984) but its proposed involvement in the nuclear accumulation of at least one of these proteins, the yeast histone H2B, is difficult to reconcile with deletion analysis which shows that this region is unimportant to its in vivo function (Wallis et al., 1983).

When the protein sequences of the NP proteins of the influenza virus used in this study (A/NT/60/68; Huddleston and Brownlee, 1982), A/PR/8/34 (Winter and Fields, 1981) and B/Singapore/222/79 (Londo et al., 1983) are aligned to satisfy the criteria of maximum amino acid homology, a highly conserved sequence within the proposed karyophilic signal-containing region is revealed (Figure 81). Although identical for the two type A NPs it may not be significant since they are highly conserved throughout their entire length. However, it represents the second most highly conserved stretch of 10 residues between type A and type B NPs differing only at residue 338 (phe→tyr), but retaining hydrophobicity. This is remarkable because type A and type B NPs not only differ in MW (56,000 and 61,600 respectively) but are antigenically totally distinct; it could therefore reflect the need to retain a

Figure 81 Comparison of the proposed karyophilic signal region of the NP of A/NT/60/68 with similar sequences from other influenza viruses

	336	338	340	342	344
A/NT/60/68	Ala	Ala	Phe Glu Asp	Leu Arg	Val Leu Ser
A/PR/8/34	Ala	Ala	Phe Glu Asp	Leu Arg	Val Leu Ser
B/Singapore/222/79	Ala	Ala	Tyr Glu Asp	Leu Arg	Val Leu Ser
	392	394	396	398	400

Amino acids are indicated by the three-letter code. The number above the amino acid indicates its position in the type A virus NP while the number below the amino acid indicates its position in B/Singapore/222/79. Type A and type B sequences are aligned on the criteria of maximum homology (Londo et al., 1983).

sequence necessary for a fundamental feature of the protein such as its accumulation in nuclei.

4. Transport of NP into oocyte nuclei

The injection of radiolabelled NP into the oocyte cytoplasm (see 'Results', Section IV) suggests that the rate at which NP enters oocyte nuclei is faster than that predicted by free diffusion through a pore with a permeant radius of 45 Å (Bonner, 1978) and would be consistent with transport by a mediated process as described for the Rana pipiens nuclear protein RNI (Feldherr et al., 1983). Such mediated transport could also explain the ~~rapid entry of other~~ ability of nuclear proteins larger than 70,000 daltons to enter nuclei (Gurdon, 1970; Bonner, 1975a, 1975b; Dingwall et al., 1982). Since this mediated transport is limited to nuclear proteins it is likely to be controlled by some feature of the proteins themselves and one may expect this feature to be identified for NP by deletion analysis. However, the observation that all mutant NP proteins, even those which failed to accumulate there, entered the nucleus rapidly, cannot be used to suggest that the mutants retain a postulated 'rapid entry' feature for two reasons: firstly because their decreased size would be expected to increase the rate of entry anyway; and secondly because in large cells like Xenopus oocytes it is not clear if the average path length taken by an injected nuclear protein is similar to that taken by the same protein when it is expressed in oocytes via injected DNA. In this latter case it is possible that the encoding mRNA, and therefore translation, are concentrated around the

nucleus (D. Drummond and A. Colman, unpublished data).

The data obtained with the NP-globin fusion proteins may reveal the presence of a third 'nuclear' signal. Although one may predict that a protein with a MW of 12,500 should rapidly cross the nuclear envelope, globin, expressed from the plasmid pTKG, remained in the oocyte cytoplasm. This is not unique since many proteins of a similar size have recently been reported to be present in only the cytoplasm of Xenopus oocytes and HeLa cells (Dabauvalle and Franke, 1984). Although these other proteins exist in the cytoplasm in a soluble form (Dabauvalle and Franke, 1984), it is not known if the same is true for globin and it is possible that the globin is prevented from entering the oocyte nucleus by being assembled into, or associated with, large particles. When globin was fused to the first 130 amino acids of NP the resulting fusion protein entered the nucleus and became evenly distributed between this compartment and the cytoplasm. This could indicate that the first 130 amino acids of NP contain information that controls the entry of proteins into nuclei. Another possibility, however, is that the addition of these amino acids destroys those features of the globin which control its karyophobic behaviour and the resulting fusion protein merely diffuses through the nuclear pores. It is not possible to distinguish between these possibilities.

The situation regarding the use of globin fusion proteins to study nuclear entry is therefore different to their use in the study of nuclear accumulation (see above) and protein segregation into intracellular membranes (Tabe et al., 1984). In the latter two cases a

specific signal is necessary for the observed effect whereas, since the majority of small particles and proteins are capable of entering nuclei (see 'General Introduction'), it could be argued that no similar signal is required for transport into the nucleus. If such a signal exists, however, it would appear to be located in the first 130 amino acids of NP.

5. Identification of karyophilic signals

In the light of the hypothesis that the mediated entry of proteins into nuclei and nuclear accumulation are different phenomena encoded in different regions of the proteins, it is instructive to review recent attempts to identify karyophilic signals in other proteins, viz. *Xenopus* nucleoplasmin, α2 protein of yeast, SV40 large-T antigen and *Xenopus* histone H1.

Selective proteolysis cleaved nucleoplasmin into two fragments (Dingwall *et al.*, 1982) of which only the smaller retained the ability to rapidly migrate into and accumulate in nuclei, the large fragment remaining in whichever cellular compartment it was microinjected. Since the MW of the latter is greater than 70,000 its inability to be transported across the nuclear envelope could indicate that it has lost the information necessary for mediated transport while the nuclear accumulation of the small fragment suggests that it not only contains this information but also the information for nuclear accumulation.

A recent study using yeast cells in which varying amounts of the yeast protein α2 were fused to a constant portion of the Escherichia coli β-galactosidase (Hall et al., 1984) is complicated by discrepancies resulting from different methods of analysis and the observation that β-galactosidase itself is capable of entering culture cell nuclei (A. E. Smith, personal communication). Nuclear accumulation of the hybrid proteins required the first 67 amino acids of α2, and results with hybrids containing less than this were consistent with their retention in the nuclear envelope. Unfortunately, the use of indirect detection methods such as enzyme assays and immunofluorescence does not rule out the possibility that the results are due to preferential degradation of the proteins in one of the cellular compartments. Such differential stability of proteins is a well documented phenomenon both in oocytes (Lane et al., 1979; Colman et al., 1981) and tissue culture cells (Capecchi et al., 1974; McGarry et al., 1983) and its effect on the apparent distribution of proteins between the cytoplasm and the nucleus has been discussed in detail by Yamaizumi et al. (1978) and Dingwall and Allan (1984).

A similar criticism can be raised against the studies involving the SV40 large-T antigen (Kalderon et al., 1984) where the cellular location was determined by immunofluorescence. The mutation of a particularly basic tract of residues in this protein resulted in either a reduced nuclear accumulation or the loss of ability to enter nuclei which may indicate that the mutated region is involved in the entry of the protein into nuclei rather than in its accumulation. The fact that mutant proteins which showed reduced accumulation also varied in the extent to which

they accumulated in nuclei of different clonally-derived sibling cells could support this interpretation. Absence of kinetic data prevents the distinction between the two possibilities.

Proteolytic fragmentation of histone H1 has located a karyophilic signal in the carboxyl-terminal domain of this protein (Dingwall and Allan, 1984). Fragments from other domains also entered the oocyte nucleus but as these were more unstable in the cytoplasm they give only a false impression of nuclear accumulation. The small size of all the fragments makes it difficult to determine whether or not their rates of nuclear entry are in any way mediated by interaction at the nuclear envelope. Hence like the NP data described above, it is not possible to identify which region of the protein is responsible for its rapid entry into nuclei (Bonner, 1975b).

6. The use of Xenopus oocytes as a surrogate system for nuclear transport

One major concern when using a heterologous system of the type exploited for these studies is that any data obtained may only reflect the system used and have no general relevance. Thus the importance of amino acids 327 to 345 of the influenza virus NP in the nuclear accumulation of this protein may be an oocyte-specific phenomenon. Since the microinjection of nucleic acids and proteins into cultured cells is now routine in many laboratories this possibility can be investigated.

Although it is often argued, perhaps wrongly, that tissue culture cells more closely resemble a 'true' eukaryotic system, I believe that there are many reasons why Xenopus oocytes will continue to be of use as a surrogate system. Firstly, for reasons mentioned above, it is always an advantage to employ more than one surrogate system. Secondly, the ease and speed with which results can be produced using the oocyte should favour its use as a supplementary system or as a testing ground for new investigations. The oocyte is capable of correctly modifying and segregating many proteins after translation following the injection of mRNA or DNA encoding the protein (see 'General Introduction') and the efficiency of protein synthesis is often sufficiently high to preclude the use of indirect detection methods which can cause problems in interpretation. Finally, and probably most importantly from the point of view of studies on nuclear accumulation, the large size of the oocyte nucleus (approximately 0.3mm in diameter) allows its rapid manual isolation.

7. Conclusion

I suggest that the nuclear accumulation of proteins may require two signals: the first allowing the protein to enter nuclei at a rate faster than that predicted from its size, and the second allowing its nuclear accumulation. It is this second signal which we have identified in the influenza virus NP.

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